Effects of Plant Viral Pathogens on Plant-Pollinator Relationships

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ABSTRACT

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I investigated aspects of a 'payback' hypothesis that postulates that virus infection triggers changes in host plants that render them more attractive to pollinators. This builds on previous work, which showed that in tomato *Cucumber mosaic virus* (CMV) infection enhanced emission of volatile organic compounds (VOCs) that attracted bumblebees and enhanced pollination of infected plants. To test if this hypothesis is tenable with other viruses and with plants other than tomato, and to determine if pollinators might derive some advantage by visiting flowers of infected plants, I used two common bean (*Phaseolus vulgaris*) varieties, and three bean-infecting viruses (a CMV bean isolate, *Bean common mosaic virus*, and *Bean common mosaic necrosis virus*). Commercially produced bumblebees (*Bombus terrestris*) and wild bees were studied, respectively, under glasshouse conditions and in the field. My data indicates that viruses can pay back susceptible hosts by attracting pollinators through changes in host-emitted VOCs and rewarding pollinators through greater nectar quantity and sucrose concentration. The enhanced pollinator attraction correlated with a recovery in seed production in virus-infected bean plants.

Virus infection delayed the onset of flowering and decreased flower numbers, but it also caused bee-perceptible changes to flower petal colour, increased nectar volumes and nectar sucrose concentration. Changes in nectar volume/concentration are likely to encourage bee visitation. Gas chromatography-mass spectrometry revealed that headspace VOCs emitted by virus-infected plants were qualitatively distinct from those of mock-inoculated plants and that virus-infected plants emitted greater quantities of VOCs. In free-choice olfactometry assays, bumblebees displayed an innate preference for VOCs emitted by non-flowering BCMNV-infected plants and

both non-flowering and flowering BCMV and CMV-infected plants over those from mock-inoculated plants. Where bumblebees showed no innate preference, as was the case for flowering BCMNV-infected plants, differential conditional assays showed that bumblebees were nevertheless able to perceive differences between the VOCs emitted by BCMNV-infected plants and mock-inoculated plants.

I examined pollination and seed production in virus-infected bean under glasshouse conditions using *B. terrestris* and in the Cambridge University Botanic Garden using CMV-infected plants exposed to naturally occurring bees. Under Garden conditions, I found that common carder bees (*B. pascuorum*) were the main bean flower pollinators, while *B. terrestris* and honey bees acted as nectar thieves. Under both conditions, virus-infected plants showed a recovery of seed numbers to levels similar to those from uninfected plants if pollinators were allowed access. These observations of virus-induced effects on plant-pollinator interactions support the idea that viruses may act mutualistically with plants by making infected plants more attractive to pollinators and suggest that pollinators may also derive benefits from visiting infected plants.

DEDICATION

I dedicate this thesis to the people I love unconditionally and who mean the world to me.

My loving husband and best friend Mr Keith Mhlanga and our children Arthur Ngonidzashe Mhlanga and Pharrell Nkosinathi Mhlanga.

My dear brother Mr Tapiwa Machingambi and sisters Mrs Christina Anthony-Byrne and Ms Molly Machingambi.

My aunt Mrs Magret Musarurwa.

My late parents Mr Wright Machingambi and Mrs Beata Machingambi, who would have been very proud of my doctoral degree.

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TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BCMV	*Bean common mosaic virus*
BCMNV	*Bean common mosaic necrosis virus*
DMNT	8-dimethyl-1,3,7-nonatriene
DNA	deoxyribonucleic acid
cDNA	complementary DNA
CP	coat protein
dNTPs	deoxynucleotide triphosphates
MEP/DOXP	mevalonate-independent pathway and the 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GC-MS	gas chromatography-mass spectrometry
HC-Pro	helper component-proteinase
HSD	honest significance test
miRNA	microRNA
NIST	National Institute of Standards and Technology
ORF	open reading frame
PCA	principal component analysis
PCR	polymerase chain reaction
PGF	plant growth facility
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription-polymerase chain reaction
SEM	scanning electron microscope
SEM	standard error of the mean

ssRNA	single stranded RNA
TAE	Tris-acid EDTA
Tris	tris(hydroxymethyl)aminomethane
TMV	tobacco mosaic virus
UV	ultraviolet
VOC	volatile organic compound
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

GENERAL INTRODUCTION

1.1 Pollinators in agriculture and natural ecosystems

Pollinators provide a key ecosystem service, which is essential for the reproduction of many wild plants, maintenance of healthy biodiversity and functional ecosystems, crop production and food security (Ashman *et al*. 2004; Aguilar *et al*. 2006; Potts *et al*. 2010; 2016a). Wind and water can pollinate flowering plants, but animals pollinate most cultivated and wild plants (Potts *et al*. 2016a). In natural ecosystems, the interaction of angiosperms and their pollinators drove evolutionary or co-evolutionary changes that eventually led to speciation as exemplified by 'pollination syndromes' (Fenster *et al*. 2004; Armbruster 2014; Glover 2014). Thus pollinator-driven floral evolution produced angiosperm diversification as evidenced by suites of floral traits associated with the attraction, utilization, and reward of a specific group of pollinators (Table 1.1) (Fenster *et al*. 2004; Armbruster 2014; Glover 2014). As an on-going process, the interaction of angiosperms and their pollinators is essential for genetic variation in plant communities, floral diversity, ecosystem conservation and stability, speciation and evolution.

The quality and yield of approximately 75% of globally important fruits, seeds, nuts and other high-quality commodity crops like oilseed rape, coffee and cocoa are directly affected by animal pollination (Klein *et al*. 2007; Aizen *et al*. 2009; Gallai *et al*. 2009; Potts *et al*. 2010; Classen *et al*. 2014; Rader *et al*. 2015; Stein *et al*. 2017). Insects are the largest group of pollinators and include bees, beetles, butterflies, flies, moths and thrips (Rader *et al*. 2015; Potts *et al*. 2016a). A smaller group of vertebrate pollinators exist (for example birds, lizards, bats, rodents and other mammals). Among insect pollinators, bees are the most important, and they pollinate more than 90% of the leading 109 global crops (Greenleaf & Kremen 2006; Klein *et al*. 2007; Winfree *et al*. 2007). To a

Table 1.1 Summary of pollination syndromes

POLLINATION VECTOR	COLOUR	SHAPE	SCENT	REWARD
BEETLE	White/cream	Dish/bowl	Fruity, quite strong	Nectar to lap, some excess pollen
FLY	White/cream/pale yellow	Dish-like or more complex	Minimal	Small amount of nectar
BEE	Blue/yellow/ultraviolet	Deep tubes, bilateral symmetry	Minimal	More nectar, some excess pollen
BUTTERFLY	Yellow/red/orange	Deep tubes with landing platforms	Minimal	Only nectar
MOTH	White	Bilateral symmetry, deep tubes	Strong, sweet at night	Plentiful nectar
BIRD	Red/orange	Pendant or bilateral and upright, tube	Minimal	Much nectar
BAT	White	Large saucer shape	Strong, butyric acid	Copious nectar and much excess pollen
WIND	Pale green, brown, or colourless	Regular and small	None	None

Table from Glover (2014) showing the general features of flowers involved in different pollination syndromes.

large extent, pollination depends on the symbiotic relationship between the pollinated and the pollinator, such that the reduction or loss of either will affect the survival of both (Kearns *et al*. 1998). Thus, the survival of insect-pollinated wild plants and crop productivity is threatened by declining bee populations (Biesmeijer *et al*. 2006; Gallai *et al*. 2009; Potts *et al*. 2010; Abrol 2011; Goulson *et al*. 2015). The decline in bee populations is partly attributable to pathogens and parasites like RNA viruses and varroa mites respectively (Genersch *et al*. 2006; Fürst *et al*. 2014; Cameron *et al*. 2011; Roberts *et al*. 2015; Wilfert *et al*. 2016), and other factors including habitat loss and fragmentation caused by land tenure systems, pollution, pesticides, introduced alien species, declining resource diversity, and climate change (reviewed in Potts *et al*. 2010; Goulson *et al*. 2015; Brown *et al*. 2016). Neonicotinoid insecticides used in agriculture have been shown to contribute significantly to declining bumblebee populations (Baron *et al*. 2017). A potential replacement to controversial neonicotinoids, sulfoximine-based insecticide sulfoxaflor, is also harmful to bumblebees (Raine 2018; Siviter *et al*. 2018). Goulson *et al*. (2015) also suggest that dietary stress, due to lack of suitable flowers, compromises the ability of bees to resist pathogens and cope with toxins and parasites.

According to Potts *et al*. (2016a), 12 out of over 20,000 bee species that have been described worldwide are commonly used as managed bees for crop pollination. Examples include honey bees (*Apis cerana* Fabricius and *Apis mellifera* L.), some bumblebees (*B. terrestris* L.), stingless bees, and solitary bees. Together with a diverse assemblage of wild pollinators, managed bees significantly contribute to global crop production (Garibaldi *et al*. 2013). Apart from crop production, honey-hunting and beekeeping practices based on indigenous and local knowledge helps to alleviate poverty among rural communities and ensure livelihood security in more than 50 countries (Crane 1999; Gupta *et al*. 2014). The importance of bee conservation has seen increased interest from science, policymakers, and the public, for better management responses (Lautenbach *et al*. 2012; Potts *et al*. 2016b).

1.2 Bumblebees in ecosystems, agricultural systems and research

Bumblebees (*Bombus* Latr.) are cold-adapted bees native to the cool temperate and cold regions of Europe and the north coast of Africa, all major Mediterranean islands (e.g. Crete, Cyprus, Sardinia), some Atlantic islands (Canary Islands and Madeira) (Estoup *et al*. 1996; Widmer *et al*. 1998; Williams 1998; Chittka *et al*. 2004), Asia (Williams 1991), North America (Williams *et al*. 2014), and South America (Plischuk *et al*. 1999). According to Heinrich (1979), the *Bombus* genus evolved thermoregulatory adaptations involving facultative endothermy, which enables them to live in some of the coldest insect-inhabited ecosystems such as highest-elevation alpines, boreal, arctic and subarctic regions. Thus, bumblebees evolved the ability to elevate thorax temperature above ambient temperature by elevating metabolism and contracting antagonistic flight muscles without moving their wings (Heinrich & Kammer 1973; Heinrich 1974; Heinrich 1979). Although some areas have native bumblebees, commercial European bumblebees have been introduced to several parts of the world as commercial pollinators including Japan (Inari *et al*. 2005), Canada (Whittington *et al*. 2004; Winter *et al*. 2006), Mexico and the United States (Winter *et al*. 2006), New Zealand (MacFarlane & Gurr 1995; Goulson & Hanley 2004), Israel (Dafni & Shmida 1996; Dafni 1998), Chile (Ruz & Herrera 2001), and Tasmania and mainland Australia (Semmens *et al*. 1993; Hingston 2005; 2006).

According to Velthuis & van Doorn (2006), *B. terrestris* can be regarded as a domesticated species because of the ability of bees in this species to easily nest in artificial nest boxes. Together with honey bees, bumblebees such as *B. terrestris audax* Harris (buff-tailed bumblebee) are utilized worldwide for pollination of several economically important crops in greenhouses and orchards (Carreck & Williams 1998; Delaplane & Mayer 2000; Albrecht *et al*. 2012). Bumblebees can be more effective than honey bees, thanks to their longer tongues and quicker foraging speed (Chittka *et al*. 2004). Furthermore, they are able to forage under rough conditions such as low temperatures and windy weather. The commercialisation of bumblebees began in Belgium with

the discovery of the benefits of using *B. terrestris* to pollinate greenhouse tomatoes compared with other pollination methods in 1985 (Velthuis & van Doom 2006). *B. terrestris* colonies are commercialised for use by farmers and research institutions as boxed ready-made shelf products that are easily transported. This facilitates bumblebee studies in their natural habitats and in the laboratory as amenable model pollinators. They are used extensively as a model system in insect sensory, behavioural ecology and pollination studies (Goulson 2003; Woodard *et al*. 2015; Groen *et al*. 2016; Jiang 2017). This is the reason why *B. terrestris audax* was chosen for my experiments in the laboratory arena and greenhouse.

Buff-tailed bumblebees are a pollen-storing species with adults that feed their larvae directly on regurgitated nectar and pollen mixes; most of the larval development occurs in individual silk cells (Alford 1975). They are generalist pollinators, and this is made possible by their ability to forage over long distances (Walther-Hellwig & Frankl 2000; Kreyer *et al.* 2004), the early seasonal emergence of their queens (Sladen 1912; Prys-Jones & Corbet 1991), and their behavioural skills for pollen and nectar gathering, which include buzz pollination and nectar robbing respectively (Prys-Jones & Corbet 1991; Proctor *et al*. 1996). Bumblebees are guided to suitable flowers by multimodal cues including visual and olfactory stimuli (Glover 2007; 2014; Katzenberger *et al*. 2013; Lawson *et al*. 2017) and can learn effectively to associate particular floral features with nectar and pollen rewards (Cnaani *et al*. 2006; Raine *et al*. 2006; Gomez *et al.* 2008; Eisenhardt 2014; Konzmann & Lunau 2014).

Bumblebees and other bee pollinator populations have been reported to be declining (see section 1.1). The deformed wing virus which is vectored by varroa mites is a major threat to *A. mellifera* and *B. terrestris* (Genersch *et al*. 2006; Fürst *et al*. 2014; Wilfert *et al*. 2016). The commercial bumblebee rearing practise of inducing colony founding by placing honey bee workers with bumblebee queens has mediated host shifting of the deformed wing virus from honey bees to

bumblebees (Genersch *et al*. 2006). The virus has also spread to wild *B. pascuorum* (Scopoli) through their behaviour of robbing managed honey bee hives (Genersch *et al*. 2006). According to Roberts *et al*. (2015), the introduction of managed bees into new areas may facilitate the introduction of RNA viruses to novel vectors. These fast-evolving RNA viruses emerge quickly in new hosts because they have a high propensity for shifting hosts (Tehel *et al*. 2016). In comparison with other *Bombus* species, *B. terrestris* remains widespread in natural environments among other short-tongued *Bombus* species. This is because it can forage at very long distances, making it less sensitive to biodiversity and environmental changes (Carvell 2002; Goulson *et al*. 2002).

1.3 Plant-pollinator relationships

Plant-pollinator interactions are good examples of mutualism, where there is an exchange of goods or services and each species involved receives a benefit from the interaction, but that benefit usually comes at a cost (Bronstein 1994). Most pollinators are rewarded with food in the form of nectar or pollen, and plants benefit through pollen transfer for reproduction. Other apoid pollinator benefits include resins and waxes from flowers used by some bees to build their hives (Michener 2007) and volatile organic compounds (VOCs) from orchid flowers used by male euglossine bees as pheromones to attract mates (Zimmermann *et al.* 2006). Other insect species like yucca moths lay their eggs within the yucca flowers they pollinate, and their developing larvae feed on some (but not all) of the seeds produced (Pellmyr 2003). According to Ollerton *et al*. (2011), 87.5% of the estimated species-level diversity of flowering plants are pollinated by animals. Thus, without animal pollinators, seed setting and reproduction could fail in many flowering plants; and without plants to provide food and other resources in the form of rewards, many animal pollinator populations would decline, with consequent knock-on effects for other species (Kearns *et al*. 1998).

Plant-pollinator relationships have attracted research attention for over a century. Charles Darwin in 1859 highlighted the potential role of pollination mutualisms in natural selection when he wrote, " . . . I can understand how a flower and a bee might slowly become, either simultaneously or one after the other, modified and adapted in the most perfect manner to each other, by the continued preservation of individuals presenting mutual and slightly favourable deviations of structure" (Darwin 1859). Studies on pollination during early days were primarily focused on the reproductive cycles of plant species and pollinators were largely ignored as important pollen vectors. These studies were either focused on the ecological processes that resulted in pollination or the evolutionary consequences of pollination dynamics. There has been a paradigm shift in recent years where current research in pollination science attempts to link ecological and evolutionary approaches to understand evolutionary processes in the ecological contexts in which they occur (Mitchell *et al.* 2009a).

Pollination syndromes (Table 1.1) are suites of floral traits that have arisen in response to selective pressures imposed by different pollen vectors that may be abiotic (wind and water) or biotic (animals) (Faegri & van der Pijl 1979; Proctor *et al*. 1996; Armbruster 2014; Glover 2014). Animal pollinators use multimodal floral trait cues to locate plant species of particular interest to them. Insect pollinators, including bees, can learn to associate the reward they get from a flower with its floral traits (Cnaani *et al*. 2006; Raine *et al*. 2006; Gomez *et al.* 2008, Eisenhardt 2014; Konzmann & Lunau 2014). This leads to preference for a unique signal associated with the highest reward in any given plant community, and hence the establishment of floral constancy by pollinators (Schiestl & Johnson 2013).

Floral traits that attract bee pollinators can be categorised as (1) general flower morphology (Harder1983; West & Laverty 1998); (2) flower colour (Raguso & Willis 2002; Chittka & Raine 2006; Glover 2011); (3) epidermal morphology (Glover & Martin 1998; Whitney *et al*. 2009;

Whitney *et al*. 2011), (4) floral volatile organic compounds profile (Kunze & Gumbert 2001; Burger *et al*. 2010; Suchet *et al*. 2011); and (5) the quality/quantity of the reward (Shafir *et al*. 1999; Cnaani *et al*. 2006; Raine *et al*. 2006; Gomez *et al.* 2008; Konzmann & Lunau 2014; Mallinger & Prasifka 2017). In combination, these floral traits are the foundation of bee pollinator partialities (Kulahci *et al*. 2008; Willmer 2011; Schiestl & Johnson 2013).

Flowers advertise themselves to pollinators from a distance through the use of brightly coloured and scented petals (Raguso & Willis 2002; Chittka & Raine 2006; Glover 2011). Some petals also have nectar guides with a different colour from the petals to assist the pollinator in quickly finding nectar, thereby reducing handling time (Waser & Price 1985; Leonard & Papaj 2011). Nectar guides have also been shown to reduce chances of nectar robbing by bumblebees as they induce bumblebees to collect nectar legitimately more frequently from flowers (Leonard *et al*. 2013). Flower-visiting hymenopteran insects have a remarkably conserved trichromatic vision (Figure 1.1). They are sensitive to ultraviolet, blue and green light (Figure 1.1). This was discovered through electrophysiological recordings from several hymenopteran species and subsequent phylogenetic analyses (Peitsch *et al*. 1992; Briscoe & Chittka 2001; Skorupski *et al*. 2007). Bees see the world in different colours from humans. Chittka (1992) modified the colour hexagon (Figure 1.2) for humans to visualise how bees see colour. Chittka's hexagon represents all the potential colours perceived by a bee and a position occupied by each colour is calculated according to proportional reflectance of each wavelength of light and excitation responses of the three hymenopteran photoreceptors.

Chittka *et al*. (1999) suggested that hymenopteran photoreceptor sensitivities antedate the evolution of angiosperms. Angiosperms from Europe and the Middle East show good evidence of a positive correlation between the evolution of flower colour and discrimination thresholds at which bees can best resolve colour differences (Chittka & Menzel 1992). Additionally, bees can reliably

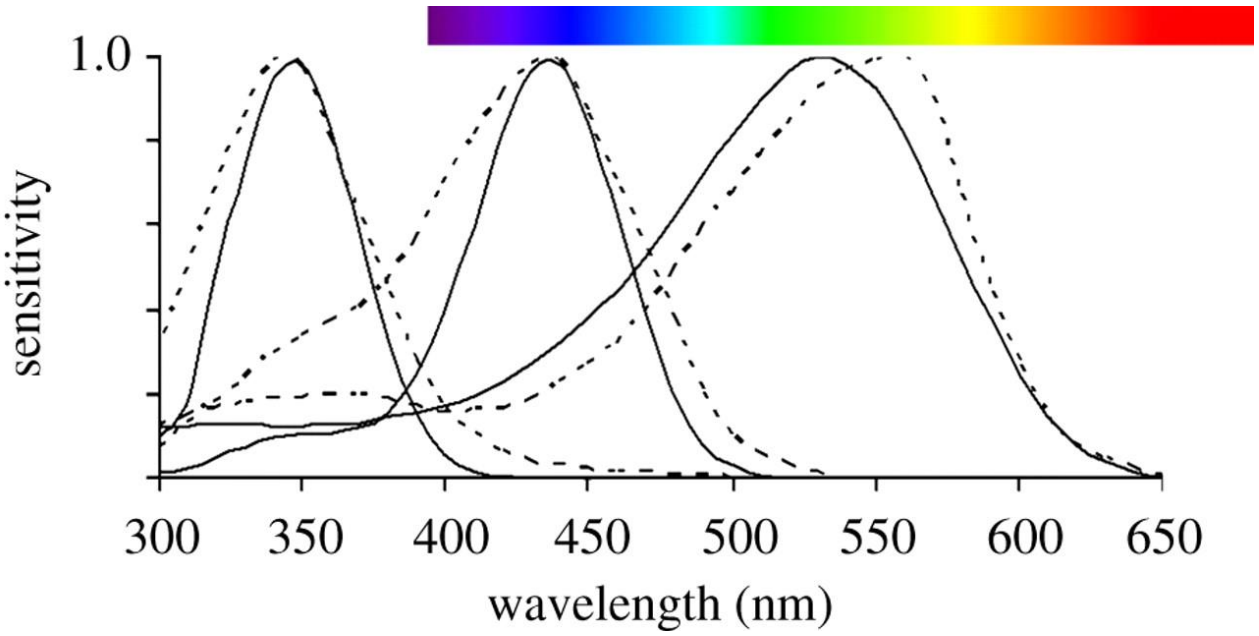


Figure 1.1 A representative of sensitivity of the three photoreceptors of bumblebees and honey bees. Many hymenopteran species are trichromatic, with an ultraviolet-sensitive (short-wavelength-sensitive, 300–400 nm), blue-sensitive (medium-wavelength-sensitive, 400–500 nm) and green-sensitive (large-wavelength-sensitive, 500–600 nm) photoreceptors. Human vision perceives longer-wavelength radiation as shown by the visible light spectral bar above the graph. Dotted line plots show honey bee photoreceptors and solid lines for bumblebees. Adapted from Dyer *et al.* (2011).

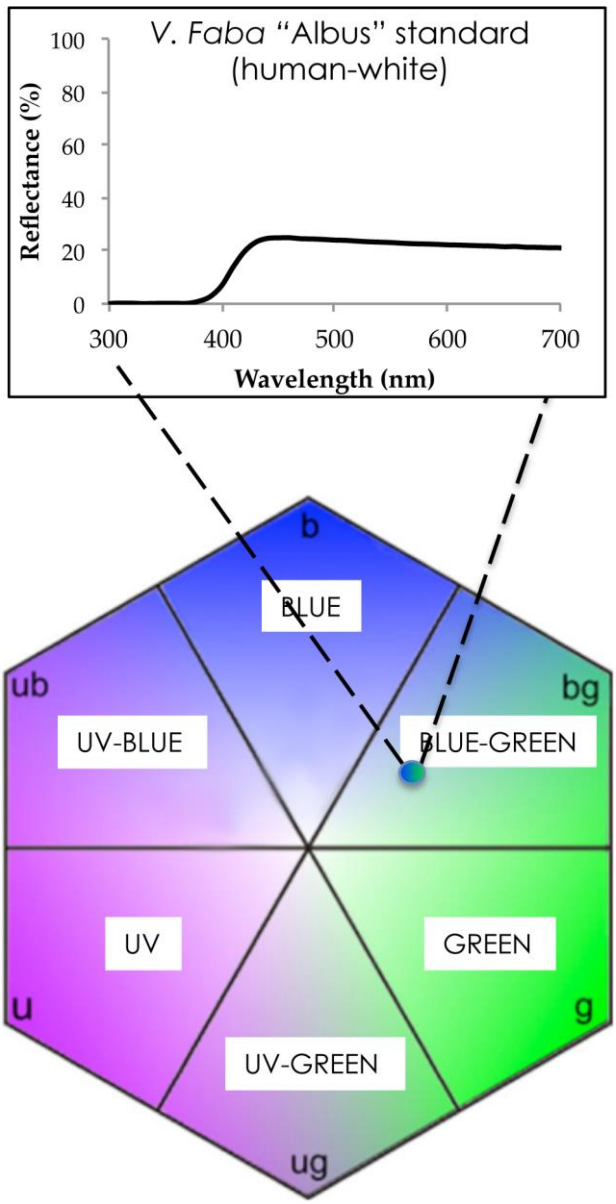


Figure 1.2 Chittka's (1992) colour hexagon representing the visual range of a bee. Points at the centre of the hexagon appear achromatic to a bee. Above the hexagon is a reflectance spectrum of the standard of *Vicia faba* "Albus" (NV643) which appears white to the human eye. A co-ordinate in the hexagon is calculated according to proportional reflectance of each wavelength of light and excitation responses of each hymenopteran photoreceptor - blue-green in this case.

discriminate colours of rewarding from non-rewarding flowers, and this could have caused the natural divergence of striking flower colours (Dyer & Chittka 2004). Red flowers are achromatic to bees, and Lunau *et al*. (2011) suggested that the avoidance of red flowers provides a 'private' niche for hummingbirds. Indeed, hummingbird pollinated flowers have acquired specific traits to exclude bees, namely: lack of a landing platform; inappropriate size of the corolla tube, and dilute nectar (Raven 1972; Irwin *et al*. 2004; Rodríguez-Gironés & Santamaría 2004).

Between 75 and 80 % of angiosperms possess conical epidermal cells on their petals (Christensen & Hansen 1998; Whitney *et al*. 2011). According to Ojeda *et al*. (2009), there are six main categories of petal epidermal cell types in the Fabaceae (Figure 1.3) based on both their primary (cell shape) and secondary structure (cell wall fine relief). These cell types were surveyed from the petal epidermal morphology of 175 legume species, representing all 12 major clades of the Fabaceae family (Figure 1.3) (Ojeda *et al*. 2009). Gorton & Vogelmann (1996) proposed that conical cells focus light onto floral pigments, thereby enhancing floral colour. Another function of conical cells as proposed by Whitney *et al*. (2009) is energetic reward to visitors by providing grip on the flower surface for easier flower manipulation and ultimately reducing the energy used on accessing the reward and in turn enhance bee pollination success (Glover & Martin 1998; Whitney *et al*. 2011). Alcorn *et al*. (2012) also suggested that the existence of conical cells in several diverse angiosperm species was a result of the natural selection of conical-celled flowers by pollinators when flowers were moving in the wind. Another proposal is that conical cells increase intrafloral temperature using solar irradiance (Comba *et al.* 2000). This warms up visiting bees, thus rewards the bees since they require high thoracic temperatures for foraging and flight between flowers (Roberts & Harrison 1999; Kovac & Stabentheiner 2011).

Flower shape, size and symmetry act as long-distance advertisements to pollinators (West & Laverty 1998). The angle at which the flower is held affects flower handling by pollinators (Harder

1983). In general, large flowers are more attractive to bee because they are more conspicuous from a distance (Goulson 1999; Duffield *et al*. 2008). Spaethe *et al*. (2001) revealed that it is substantially harder for bees to detect smaller flowers because this requires longer search times and decreases foraging efficiency. In contrast, pollinators with short tongues, like hoverflies and beetles, are attracted to smaller flowers because they have easier access to pollen and nectar (Colley & Luna 2000). Nonetheless, in some cases, large flowers may be more complex and more difficult to handle (Westerkamp 1997).

Bees learn effectively to associate specific floral features with better dietary rewards (Raine *et al*. 2006; Gomez *et al*. 2008). Bees have a limited diet that consists of nectar (carbohydrates source) and pollen (providing lipids, minerals, vitamins and the principal source of protein) (Haydak 1970; Vaudo *et al*. 2015). Chances of a bee revisiting plants of a specific species depend on the quality and quantity of the rewards. In a study by Raine *et al*. (2006), laboratory-reared *B. terrestris audax* were presented with blue non-rewarding and yellow-rewarding artificial flowers. The bees initially preferred non-rewarding blue flowers, but their preference changed completely after probing rewarding yellow flowers (Raine *et al*. 2006). Raine *et al.* (2006) explained the initial unlearned preference for blue flowers based on the assumption that blue flowers are generally profitable to *B. terrestris audax* in the British foraging environment as evidenced by preference for blue flowers in all bumblebee species (Chittka & Wells 2004).

Gomez *et al*. (2008) investigated whether some traits that are under pollinator-mediated selection are associated with nectar and pollen reward in a Mediterranean herb *Erysimum mediohispanicum* Polatschek (Brassicaceae). They found a significant correlation between corolla tube length and nectar production rate, and between corolla diameter and pollen production. Large bees and butterflies visited larger flowers with longer corolla tubes more often, and this observation was significant (Gomez *et al*. 2008). Several studies have also shown that

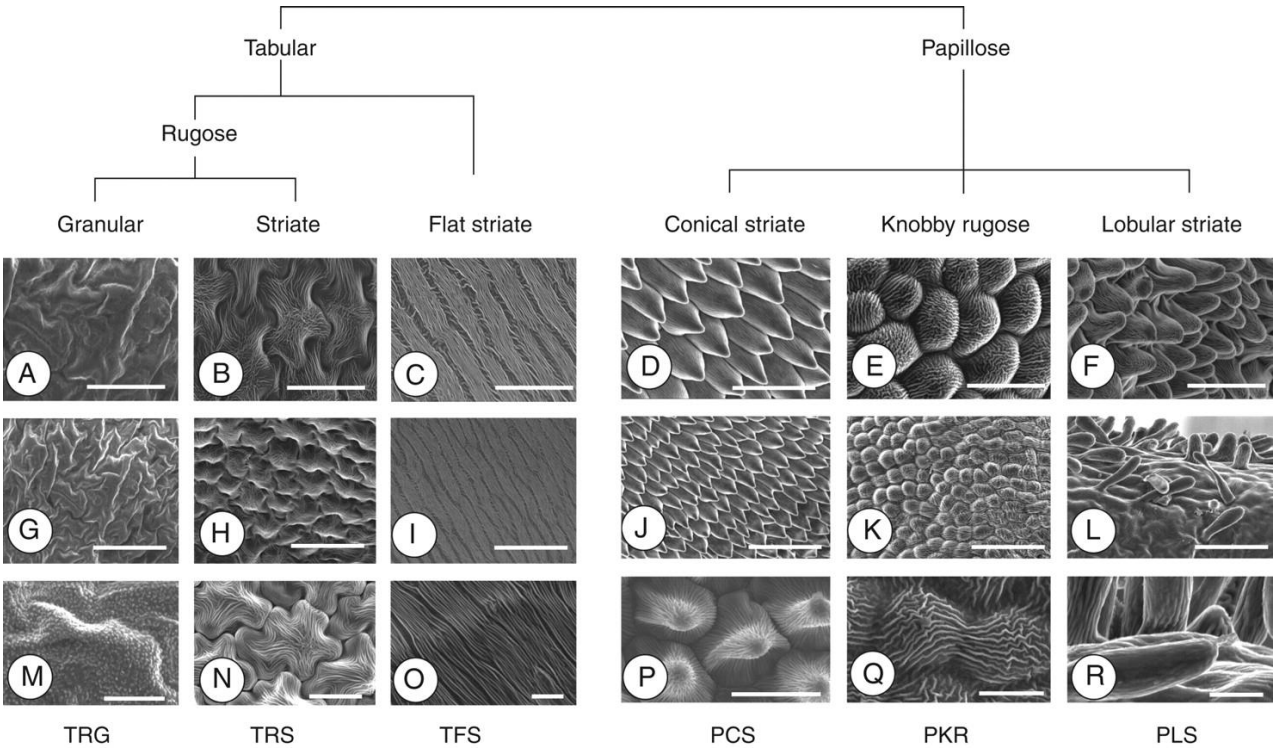


Figure 1.3 Types of petal epidermal cells found in legumes. Scale bars: (A–F) = 50 µm; (G–L) = 100 µm; (M–R) = 20 µm. Adapted from Ojeda *et al*. (2009).

bees are deterred by low reward encounters (Real 1981; Waddington *et al*. 1981; Shafir *et al*. 1999). Bumblebees show stronger preferences for flowers with high concentration and volume of nectar biased towards high sucrose concentration (Cnaani *et al*. 2006; Konzmann & Lunau 2014). According to Robertson *et al*. (1999), bumblebees prefer pollen grains that are viable in contrast to honey bees that have been shown by Pernal & Currie (2002) to have no such preferences and are instead attracted by pollen odour.

VOCs act as long-distance signals for pollinators to locate flowers (Kunze & Gumbert 2001; Burger *et al*. 2010; Suchet *et al*. 2011) and can trigger an immediate decision to land on a flower when the pollinator is close by (Lunau 1992). Bees have a poor visual resolution (Chittka & Raine 2006) and hence rely on such signals, among others. Bees also use VOCs to distinguish between rewarding and non-rewarding flowers of the same species (Dobson & Bergstrom 2000; Howell & Alarcon 2007). Free-foraging *Osmia* Panzer (Hymenoptera: Megachilidae) bees were shown to have the ability to differentiate nectar rewarding *Penstemon caesius* A. Gray flowers from nectar-depleted ones using VOCs which explained observations made of more frequent visits to flowers with nectar (Howell & Alarcon 2007). To prove that bees were using VOCs to detect the presence of nectar, Howell & Alarcon (2007) compared floral preference of bees with antennae covered with nontoxic silicone and bees with uncovered antennae. The bees were presented with nectar-depleted flowers with added water, nectar-rewarding flowers and nectar-depleted flowers of *P. caesius* (Howell & Alarcon 2007). Bees with antennae covered with silicone visited all *Pestonum* flower arrays equally whereas bees with uncovered antennae visited nectar-rewarding flowers twice as much as they did either group of nectar-depleted flowers (Howell & Alarcon 2007).

1.3.1 Model plants for the present study

During my studies, I used plants of common bean (*Phaseolus vulgaris* L.) (see Section 2.2.1) in

my experiments. It is one of the most widely grown legumes in the world. Cultivars of common bean have a worldwide distribution and are useful to mankind as sources of food, stock feeds, ornaments, medicine and organic fertilizers (Duranti & Gius 1997; Duc *et al*. 1999; Sauvant *et al*. 2004; Gepts *et al*. 2008). Poorer countries in Africa and Latin America use the crop for direct human consumption because of its high nutritional value (Duc 1997; Broughton *et al.* 2003). They are beneficial to agriculture because of their ability to host nitrogen-fixing bacteria (Duc 1997; Köpke & Nemecek 2010). Two main forms of common bean are grown all over the world the dry bean (seeds harvested) and the snap bean (green pods harvested). The two forms combined account for about 45 million hectares under bean cultivation globally (FAO 2014). Common bean belongs to the Fabaceae family and Papilionoidae subfamily. It has typical zygomorphic papilionaceous flowers (irregular and butterfly-like corolla) which consist of five petals: one standard (the posterior petal); two wings (the lateral petals), and two coiled keels (the two lower fused anterior petals) (Figure 1.4) (Duc 1997; Nassar *et al*. 2010; Aronne *et al*. 2012). There are ten stamens, nine of which are with fused filaments from the base to nearly more than half of their length while the anthers are free (Aronne *et al*. 2012). This enables the stigma to extend beyond the anthers during visitations by bees, thereby avoiding self-pollination (Lavin & Delgado 1990). The posterior stamen is free; hence the androecium is diadelphous (Aronne *et al*. 2012). Both the androecium and pistil are enclosed in a pair of fused keel petals (Figure 1.4) (Aronne *et al*. 2012). These reproductive structures are protected from biotic and abiotic factors like insects and rainfall respectively and thus during pollination pollen is deposited on body parts of bees on positions difficult for the bees to brush off (Westerkamp 1997). Common bean flowers can be white, pink, purple or red, depending on the cultivar (Graham & Ranalli 1997).

P. vulgaris is cross-fertilised, although self-fertilisation also occurs (Poulsen 1975; Triana *et al*. 1993; Svendsen & Brodsgaard 1997). Floral morphology of this species requires, almost exclusively, Hymenopteran pollinators (i.e. bees and certain wasps) with sufficient weight and

strength to mechanically “trip” the hull and wings to expose the stamens and pistil so that they can forage on the pollen/nectar (Aouar-Sadli *et al*. 2008). During flower visitations by bees, if sufficient pressure is exerted on the wings and standard petals, this will push the stigma past the keel petals and into contact with the bee to receive pollen (Galloni *et al*. 2007; Aronne *et al*. 2012). The style protrudes immediately following the stigma; it brushes pollen on the bee’s thorax (Galloni *et al*. 2007; Aronne *et al*. 2012). The style and stigma return inside the keel petals when pressure is released and the wing petals return to their former positions as well (Aronne *et al*. 2012). This brush mechanism can be repeated several times to exhaust all the pollen on the anthers (Galloni *et al*. 2007). Researchers have demonstrated and recognised the importance of bees in the cross-pollination of bean plants and the improvement of their production (Singh & Bhatt 2012). In Europe, pollinators of common bean have been identified as bumblebees, honey bees and solitary bees (Stoddard & Bond 1987; Varis 1996; Pierre *et al.* 1997). Bumblebees have been cited as the best bean plant pollinators because of their hairy bodies, weight and speed in flight (Pierre *et al*. 1997).

The origin of common bean was recently resolved by Bitocchi *et al*. (2012) as being Mesoamerican, most likely located in a region within what is now modern Mexico. Cultivars of common bean have a worldwide distribution and are useful to mankind as sources of food, stock feeds, ornaments, medicine and organic fertilizers (Gepts *et al*. 2008; Worrall *et al*. 2015). In poorer countries in Africa and Latin America direct human consumption of the crop is more important than richer countries because of its high nutritional value (Broughton *et al.* 2003; Worrall *et al*. 2015). Many common bean cultivars are susceptible to viral pathogens, including BCMV, BCMNV (Worrall *et al*. 2015) and certain strains of CMV (Morales 2006). A ground-breaking discovery was made by Ralph Corbett in the early 1930s; the discovery of resistance to viral infections of the ‘Corbett Refugee’ variety of *P. vulgaris* conferred by the *I* gene (Pierce 1934). Since then, this resistance gene has been incorporated into bean cultivars worldwide (Mavric &

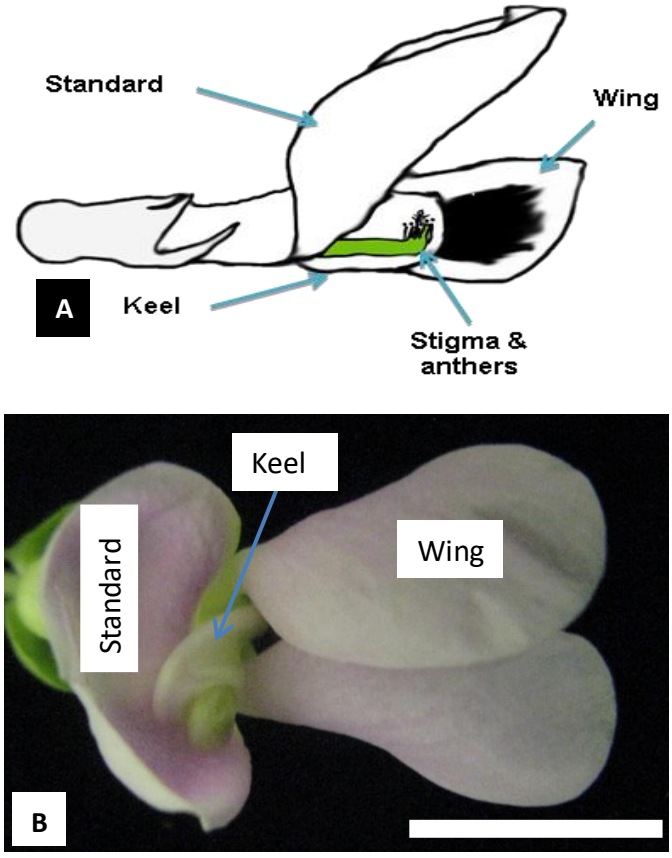


Figure 1.4 Typical papilionaceous flowers. Panel (A) shows a diagram of a *Vicia faba* flower and (B) shows a picture of a *Phaseolus vulgaris* flower with visible coiled keel petals. The floral architecture of *V. faba* and *P. vulgaris* is fundamentally identical. Diagram courtesy of Dr Emily Bailes. Scale bar B = 1 cm.

Sustar-Vozlic 2004). However, in the late 1930s, a new disease emerged referred to as the 'Black Root,' and it could overcome the resistance conferred by the *I* gene (Jenkins 1940). Symptoms of this disease as described by Jenkins (1940) include severe wilt associated with chlorosis of lower leaves and necrotic streaks parallel to the stem length. Follow up research has shown that development of symptoms is associated with the *I* locus in the presence of different legume-infecting potyviruses and different temperature ranges (McKern *et al.* 1992; Fisher & Kyle 1994; Mavric & Sustar-Vozlic 2004). The virus causing black root was named BCMNV (see Section 1.4.1.2). At 25°C, no notable symptoms occur from infections of potyviruses like BCMV, but at temperatures above 30°C, systematic veinal necrosis results due to failure of the resistance (Fisher & Kyle 1994; Mavric & Sustar-Vozlic 2004). Infection by BCMNV causes resistance failure regardless of temperature (McKern *et al.* 1992; Silbernagel *et al*. 2001; Mavric & Sustar-Vozlic 2004).

1.4 An overview of plant viruses

Many plant viruses are vectored by arthropods, nematodes, fungi, and plasmodiophorids. Among arthropod vectors, aphids are the most common vectors vectoring more than 550 virus species (Andret-Link & Fuchs 2005). The majority of plant viruses have RNA genomes and have some degree of sequence identity with insect or even vertebrate-infecting viruses suggesting insects provide a bridge for the evolution of viruses from being animal to plant viruses and vice versa (Roossinck 2003). Certain double-stranded RNA viruses are often found in fungi and plants. They have a persistent lifestyle and are transmitted vertically through gametes, but do not cause apparent symptoms in most cases (Milne & Marzachi 1999).

One of the earliest documented observations of plant viral disease is said to date back to at least 752 AD. This suggestion is based on a poem written by the Empress Koken in Japanese, which describes plants with yellowing leaves, now recognised to be typical of virus symptoms (Hull

2002). From about 1600 to 1660, people in Western Europe made several drawings and paintings of tulip flowers that demonstrate virus symptoms (Lesnaw & Ghabrial 2000; Hull 2002). Scientific investigations on plant viruses began in the 1880s and 90s with work by Dmitri Iosifovich Ivanovsky and Adolf Mayer (Zaitlin 1998). In 1898 M.W. Beijerinck produced the first definition of a virus "*contagium vivum fluidum"* on tobacco (Creager *et al*. 1999; Hull 2002). The infectious agent as a novel type and the agent worked on by these pioneers was later named tobacco mosaic virus (TMV) (Zaitlin 1998; Creager *et al*. 1999; Hull 2002). TMV has been at the forefront of virology research as a model system to the present time (Creager *et al*. 1999; Hull 2002). Much of our knowledge of plant–virus interactions comes from research based on economically important crops. More recent work has also used the model plant *Arabidopsis thaliana* (L.) Heynh. Virus infections can reduce plant growth in crops by manipulating metabolism and defence mechanisms, depressing photosynthesis and altering resource allocation (Técsi *et al*. 1996; Ziebell *et al*. 2011; Palukaitis *et al*. 2013; Alexander & Cilia 2016). Statistics from the last decade show that South-East Asia lost over $1.5 billion per year in rice production and over £20 million per year was lost in the United Kingdom in potato production because of plant virus diseases (Hull 2002). Several authors have reported up to 100% losses in yields of legumes due to BCMV, BCMNV and CMV, among other viruses (Morales 2006; Worrall *et al*. 2015).

Viruses can induce colour changes in flowers, for example, colour breaking; changes in usual pigment pattern of the perianth into irregular patches or streaks of pigmentation (Hunter *et al*. 2011). Dekker *et al*. (1993) identified five potyviruses that cause flower colour breaking in tulip and lily, and Hunter *et al*. (2011) found narcissus mosaic virus to be associated with colour break in reverse bicolour in daffodils. Between 1634 and 1637, colour-broken tulips were so popular in the Netherlands that extraordinarily high prices were paid by financial speculators (Lesnaw & Ghabrial 2000; Hull 2002). The colour-broken tulips were highly sought after because of their beauty and rarity, and this resulted in a phenomenon known as 'Tulipomania' (Lesnaw & Ghabrial

2000; Hull 2002). Unfortunately, the infected flowers are no longer desirable because the viral infection often reduces plant health and vigour, leading to economic losses in the ornamental industry (Lesnaw & Ghabrial 2000; Hull 2002). On the brighter side of colour breaking induced by viruses, as indicated in Section 1.3, flower colour affects pollinator preference, and it is possible that the new colour patterns in colour broken flowers could result in the attraction of more or different pollinators (see later Sections).

1.4.1 Viruses used in this study

1.4.1.1 Cucumber mosaic virus

CMV is the type species of the genus *Cucumovirus* within the *Bromoviridae* family (reviewed by Roossinck *et al*. 1999; Jacquemond 2012). The CMV genome is made up of three single-stranded positive-sense RNA molecules: RNA 1, RNA 2 and RNA 3 (Figure 1.5) (Palukaitis *et al*. 1992; Jacquemond 2012). During replication, additional sub-genomic RNA molecules are generated including RNA 4, which is derived from RNA 3 and encodes the coat protein (CP) (Jacquemond 2012) and RNA 4A, derived from RNA 2, which encodes the 2b protein (Figure 1.5) (Ding *et al*. 1994; Mayers *et al*. 2000). RNA 1 encodes the 1a protein, and RNA 2 encodes the 2a protein (Figure 1.5), and these proteins play a role in forming the CMV replication complex along with other unidentified host proteins and plant tonoplast intrinsic proteins (Nitta *et al*. 1988; Hayes & Buck 1990; Kim *et al*. 2006; Jacquemond 2012). RNA 2 also encodes the 12 kDa 2b protein, the smallest protein encoded by CMV, which is expressed from an open reading frame (ORF) overlapping the 3'-terminal part of ORF 2a (Figure 1.5) (Jacquemond 2012). The 2b protein inhibits RNA silencing (Diaz-Pendon *et al*. 2007; Goto *et al*. 2007; Gonzalez *et al*. 2010, 2012; Westwood *et al*. 2013b), affects virus movement (Ding *et al*. 1995; Soards *et al*. 2002) and can induce symptoms (Ding *et al*. 1995; Lewsey *et al*. 2007). The 2b protein also inhibits salicylic acid-mediated defence responses and jasmonate-regulated gene expression (Ji & Ding 2001; Lewsey *et al*. 2010; Zhou *et al*. 2014). The 2b protein modifies plant-aphid interactions (Ziebell *et al*. 2011;

Westwood *et al*. 2013a, b) and plant-bumblebee interactions (Groen *et al*. 2016; Jiang 2017). RNA 3 encodes the 3a movement proteins (MP) (Figure 1.5) responsible for movement (Suzuki *et al*. 1991). All CMV genomic RNAs have a 5' cap structure and have conserved 3' untranslated regions terminating in a tRNA-like structure (Jacquemond 2012).

CMV is an economically important virus, which has one of the widest host range of any known virus, exceeding 1,200 plant species in over 100 families including food crops, vegetables, ornamentals and wild plants (Palukaitis *et al*. 1992; Palukaitis & Garcia-Arenal 2003; Du *et al*. 2008; Jacquemond 2012). CMV has a worldwide distribution and is primarily vectored by over 75 species of aphids in a non-persistent manner (Palukaitis *et al*. 1992; Palukaitis & Garcia-Arenal 2003; Du *et al*. 2008; Jacquemond 2012). Non-persistently transmitted viruses are not retained by the insect but are loosely bound to receptors within the insect stylet. This way, they are quickly acquired and lost/ transmitted during insect foraging (reviewed in Dietzgen *et al*. 2016). CMV strains are categorized into Subgroups I and II, and Subgroup I is further divided into IA and IB based on RNA sequence variations (Palukaitis *et al*. 1992; Roossinck *et al*. 1999; Palukaitis & García-Arenal 2003). Depending upon the host, CMV can cause a range of disease symptoms including chlorosis, epinasty, filiformism, necrotic lesions and stunting symptoms (Palukaitis *et al*. 1992; Shintaku *et al*. 1992; Suzuki *et al*. 1995; Szilassy *et al*. 1999; Diveki *et al*. 2004; Du *et al*. 2008). Subgroups IA and IB have been reported to be more virulent that subgroup II strains (Wahyuni *et al*. 1992; Zhang *et al*. 1994; Cillo *et al*. 2009).

1.4.1.2 Bean common mosaic virus (BCMV) and Bean common mosaic necrosis virus (BCMNV)

BCMV and BCMNV belong to the *Potyviridae* family. Potyviruses have single-stranded positive-sense RNA genomes (reviewed in Worrall *et al*. 2015). They have rod-shaped non-enveloped flexuous particles 11-13 nm in diameter and 680-900 nm long (Oana *et al*. 2009). Genomes of

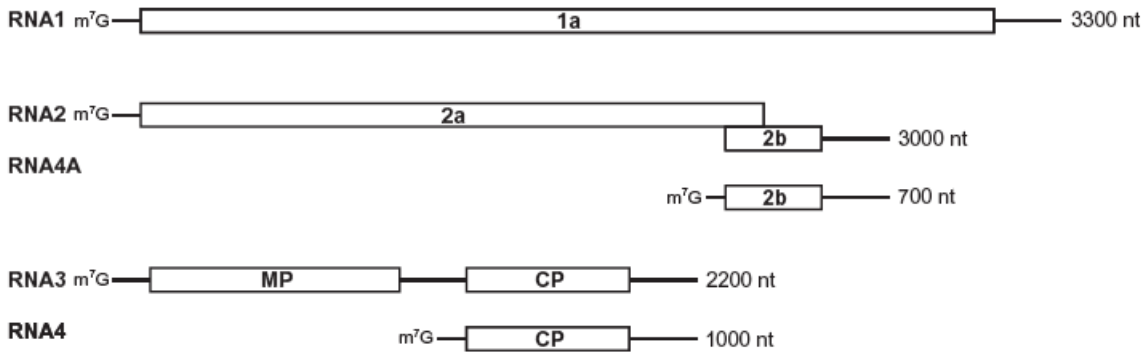


Figure 1.5 CMV genomic organization. Nucleotide (nt) numbers correspond to approximate sizes. RNA1 is monocistronic and codes for protein 1a, which possesses a putative methyltransferase domain in its N terminal domain and a helicase motif in the C-terminal domain. RNA2 encodes 2a which possesses the GDD motif typical for a viral RNA polymerase and 2b which is encoded by an ORF overlapping the 3'-terminal part of the 2a ORF. RNA3 is bicistronic and encodes the MP and the CP. The subgenomic RNAs 4A and 4 express the 2b protein and CP, respectively. The viral RNAs possess 5' m^7G cap structures. The ORFs are indicated by boxes and named according to the proteins they encode. Adapted from Jacquemond (2012).

potyviruses encode 11 functional proteins (Figure 1.6), 10 of which are derived by proteolysis from a single polyprotein precursor while the remaining one, P3N-PIPO, is generated from a short ORF embedded within the P3 cistron (Figure 1.6) (Chung *et al*. 2008). PIPO (*P*retty *I*nteresting *P*otyviridae *O*RF) is a highly conserved short ORF. It is embedded within the P3 cistron of all potyviruses and was discovered by Chung *et al.* (2008). The expression mechanism remained unknown until recently when it was shown to be caused by introduction of an additional 'A' residue into a highly conserved GAAAAAA sequence, through polymerase slippage during transcription of nascent positive strand RNA on the minus strand template, thereby enabling expression of the P3N-PIPO fusion protein (Rodamilans *et al*. 2015; Olspert *et al*. 2015; 2016). The 11 known functional proteins are listed in Table 1.2.

BCMV and BCMNV were initially treated as one species until they were shown to be separate species by McKern *et al*. (1992), Mink & Silbernagel (1992), and Vetten *et al*. (1992). Their separateness was confirmed by Mink *et al*. (1994). Vetten and colleagues (1992) showed that the BCMV (old designation: Serogroup B) genome was 10Kb and that it encoded a coat protein (CP) with a molecular weight of 35 kDa. Meanwhile, BCMNV (old designation: Serogroup A) was shown to have a genomic RNA of 9.8Kb with a CP of molecular weight of 33 kDa (Vetten *et al*. 1992). Subsequent phylogenetic analysis based on the coat protein ORF sequences and 3' untranslated region sequences further confirmed that BCMV and BCMNV were distinct virus species (Berger *et al*. 1997). Differences between BCMV and BCMNV are summarised in Table 1.3. BCMV infects legumes worldwide and is thought to have its origins in East or South Asia (Gibbs *et al*. 2008). BCMNV probably evolved from BCMV, most probably in Central or Eastern Africa (Spence & Walkey 1995; Gibbs *et al*. 2008). Hence BCMNV has a less extensive distribution than BCMV (reviewed by Worrall *et al*. 2015). Both viruses are vectored by aphids or transmitted through infected seed (reviewed in Worrall *et al*. 2015). It has, however, been noted that the success of seed transmission relies on the interaction of various factors including the

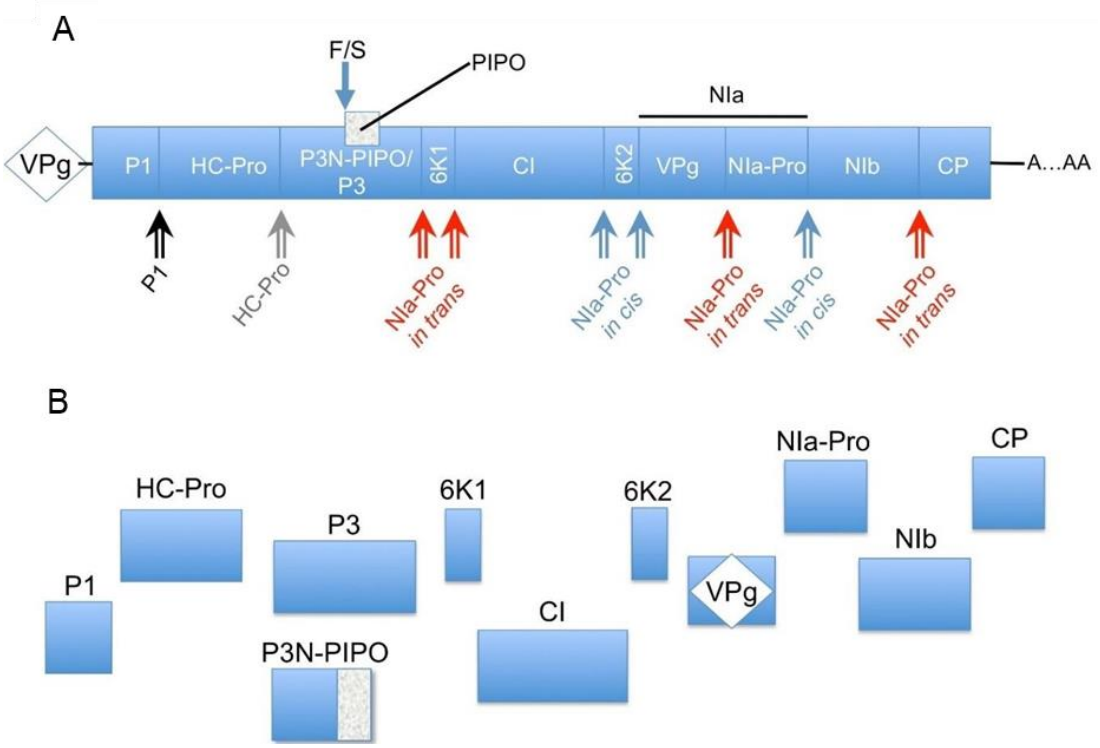


Figure 1.6 The positive-sense single-stranded RNA Potyvirus genome and synthesis of potyviral proteins. The large blue box (A) represents an open reading frame encoding a polyprotein that self-processes to produce the mature proteins (B). (A) A VPg molecule is attached to the 5'-terminal of the viral RNA in place of the 5' cap typically found on the cellular mRNAs. The RNA is 3' polyadenylated. P1, HC-Pro, and NIa-Pro are proteinases. HC-Pro cleaves in *cis* (grey arrow) to release a P1/HC-Pro fusion protein (not shown) processed in *cis* by P1 to yield P1 and HC-Pro, the mature proteins. NIa-Pro cleaves in *cis* and in *trans*. NIa-Pro *cis* cleavage activity (blue arrows) releases 6K2 and VPg/NIa-Pro fusion protein. *Trans* cleavage (red arrows) releases five proteins; mature VPg, CP, the NIb (the RNA-directed RNA polymerase), P3 and 6K1. Transcriptional slippage during viral RNA synthesis produces a small number of viral RNAs with an additional nucleotide inserted that allows expression of the PIPO ORF (grey arrow) to yield the P3N-PIPO fusion protein. Adapted from Worrall *et al*. (2015). The known roles of these proteins are listed in Table 1.2.

Table 1.2 The 11 known proteins of potyviruses adopted from Riechmann (1992), Oana *et al*. (2009), Wei *et al*. (2010) and Valli *et al*. (2014).

PROTEIN	FUNCTION(S)
P1	Genome amplification, binding RNA and symptomatology
Helper Component Proteinase (HC-Pro)	Aphid transmission, cell-to-cell long-distance movement, proteolytic activity, genome amplification, self-interaction, enhancement of pathogenicity, long-distance movement of other viruses in mixed infections, suppression of gene silencing, synergism and symptom expression, papain-like cysteine proteinase, binding RNA and C-terminal auto cleavage
P3	Pathogenicity, genome replication
6K1	Pathogenicity together with the C-terminal region of P3, genome amplification
CI	Cell-to-cell movement, ATPase/RNA helicase, genome amplification, binding RNA
6K2	Anchoring the viral replication complex to membranes, genome amplification
VPg	Genome replication, translation, movement, determination of recessive resistance
NIa	Cellular localization, trypsin-like serine protease (acts in *cis* and in *trans*), binding RNA, genome amplification, protein-protein interaction
Nib	RNA-dependent RNA polymerase (RdRp), virus assembly
CP	Aphid transmission, cell-to-cell and systemic movement, genome amplification.
P3N-PIPO	Cell-to-cell movement

Table 1.3 Difference between BCMV and BCMNV adapted from Mavric & Sustar-Vozlic (2004).

BCMV	BCMNV
serotype B	serotype A
normal length 847-886 nm	normal length 810-818 nm
CP of 34.5-35 kDa	CP of about 33 kDa
mosaic symptoms, only some strains can cause systemic lethal necrosis in sensitive cultivars at higher temperatures	systemic lethal necrosis on bean genotypes possessing dominant resistance *I* gene both at lower and higher temperatures
only typical potyvirus pinwheel and scroll inclusions in cytoplasm	a specific type of proliferated endoplasmic reticulum
sequence differences in CP and 3' non-coding region	sequence differences in CP and 3' non-coding region

environment, stage of infection, host cultivar and virus strain (Worrall *et al.* 2015). Both viruses infect wild legumes and cause severe crop losses, poor yields, and economic losses in bean production (Morales & Bos 1988; Bos & Gibbs 1995; Spence & Walkey 1995; Saqib *et al*. 2005, 2010; Morales, 2006; Coutts *et al*. 2011).

BCMV and BCMNV are transmitted by aphid vectors, including *Myzus persicae* (Sulzer) and *Aphis fabae* Scopoli (Kennedy *et al*. 1962; Halbert *et al*. 1994). BCMV symptoms depend on the host cultivar and can be affected by environmental conditions. Symptoms include mosaic patterns on leaves, malformation of leaves and pods, rugose lower leaves, and in severe cases, black root and plant mortality (George 2014). In sensitive cultivars, BCMV can cause lethal necrosis at high temperatures (Mavric & Susta-Vozlic 2004). BCMNV symptoms include leaf distortion, mosaic and stunting (Mink & Silbernagel 1992). Both low and higher temperatures can cause systemic lethal necrosis in bean cultivars carrying the dominant BCMV resistance gene *I* (Silbernagel *et al*. 2001). Nevertheless, in bean genotypes lacking resistance genes, all known BCMV and BCMNV strains cause similar symptoms (Morales & Bos 1988).

1.5 Virus-induced biochemical changes in hosts

Viruses alter host plant phenotypes, and in turn, this can affect the performance and behaviour of insects that interact with infected hosts. Researchers have shown that virus infection induces changes in VOC blend emissions and that these alterations may attract and/or repel herbivores, such as aphids and other virus vectors (Fereres & Moreno 2009; Mauck *et al*. 2010; 2012; Westwood *et al*. 2013a, Tungadi *et al*. 2017), or attract pollinators, such as bumblebees (Groen *et al*. 2016; Jiang 2017). There is a variation in preferences of aphids for virus-infected plants; aphids may or may not prefer virus-infected plants (Fereres & Moreno 2009). Westwood *et al*. (2013a) observed that CMV infection induced feeding deterrence in *Arabidopsis thaliana*. This inhibited prolonged feeding and encourages virus transmission (A. Bravo, unpublished). A

deceptive attraction of aphids (*M. persicae* and *Aphis gossypii* Glover) to virus-infected plants was observed in CMV-infected squash (*Cucurbita pepo* L.) (Mauck *et al*. 2010). The plants emitted elevated levels of volatile cues that attracted aphids, but the plants were distasteful to the insects (Mauck *et al*. 2010). Tungadi *et al*. (2017) showed that CMV increased VOC emission by tobacco plants, but the settling behaviour of aphid vectors was not affected.

Aphids colonize plants in a sequence of events in response to a variety of olfactory, visual, tactile and taste stimuli (Niemeyer 1990; Powell *et al*. 2006). The initial stage of colonization is host habitat and habitat location guided by chemical cues (Niemeyer 1990; Pickett *et al*. 1992) as well as visual cues (Kennedy *et al*. 1962; Doring *et al*. 2004). Assessment of chemical and physical leaf properties, as well as odours, follows soon after landing (Niemeyer 1990). Aphids briefly assess the quality of the host plant by sampling the gustative cues in the mesophyll cells using their stylets (Powell & Hardie 2000). If the host is acceptable, aphids will begin ingesting phloem sap (Caillaud 1999; MacKay & Downer 1979; Niemeyer 1990; Powell & Hardie 2000; Powell *et al*. 2006). If gustatory cues indicate unsuitable or distasteful host aphids move on to another plant (Powell & Hardie 2000).

Groen *et al*. (2016) showed that CMV-Fny altered the VOCs emitted by tomato plants (*Solanum lycopersicum* L.), making CMV-infected tomato plants more attractive to bumblebees. When they repeated the same experiment with a CMV mutant unable to express the 2b protein, an RNA silencing suppressor, infected tomato plants were no longer attractive to bumblebees. Their suggestion was that the 2b protein plays a vital role in re-programming VOC emission by virus-infected plants. They carried out pollination experiments in the glasshouse and discovered that bumblebee pollination rescued seed production in CMV-infected tomato plants to match with seed production in non-buzzed flowers of mock-inoculated plants. Supported by mathematical modelling, they suggested that viruses might ‘pay back’ their susceptible hosts by making their

hosts more attractive to pollinators, thereby promoting reproduction and survival of alleles for virus susceptibility. The work contributes to a growing view that viruses are mutualists under some circumstances rather than antagonists (Groen *et al*. 2016).

1.6 Viruses as mutualists

Most studies on plant viruses are biased towards the pathogenic properties of plant viruses, and thus the potentially mutualistic symbiotic properties of plant viruses have been overshadowed (Roossinck 2003; 2011). As early as 1980, Gibbs grew healthy and *Kennedya yellow mosaic virus*-infected *Kennedya rubicunda* (Schneev.) Vent. seedlings in their natural environment. He observed that herbivores grazed on healthy plants more frequently in comparison with virus-infected plants, leading to the quick disappearance of healthy plants (Gibbs 1980). He went on to do palatability trials using grated carrot bait and caged young rabbits. The caged young rabbits showed an innate preference for bait mixed with powdered virus-free *K. rubicunda* leaves over the virus-infected leaf powder and grated carrot mixture (Gibbs 1980). Van Molken *et al*. (2012) demonstrated the ability of the *White clover mosaic virus* (WClMV) to protect their *Trifolium repens* L. (white clover) hosts by decreasing herbivore infestation rates by adult fungus gnat females. They found out that WClMV-infected white clover plants were emitting β-caryophyllene which deterred the adult fungus gnat females (Van Molken *et al*. 2012). Thus, it is evident that plant viruses play a more beneficial role in plant-herbivore interactions than generally assumed.

Viruses were also shown to improve plant tolerance to abiotic stresses of drought and cold temperatures (Xu *et al*. 2008). Several species were inoculated with four different RNA viruses, *Brome mosaic virus* (BMV), CMV, *Tobacco mosaic virus* and *Tobacco rattle virus* showed drought resistance (Xu *et al*. 2008). The drought tolerance was attributed to increases in osmoprotectant and antioxidant in BMV-infected rice and CMV-infected beet plants. CMV-infected beet plants also showed induced freezing tolerance (Xu *et al*. 2008). CMV also induced drought tolerance in

Arabidopsis thaliana (Westwood *et al*. 2013b). Drought tolerance was attributed to the CMV 2b protein because *2b* transgenic *A. thaliana* plants endured drought more effectively than non-transgenic plants, and *A. thaliana* infected with a viral mutant lacking the *2b* gene, CMVΔ2b, showed no increase in drought resistance (Westwood *et al*. 2013b). Groen *et al*. (2016) showed how viruses induced host attractiveness to pollinators, thereby increasing host reproductive fitness. Taken together, these drought-resistance and improved reproductive fitness phenomena are conditional phenotypes induced by viruses that may be a form of payback to the hosts; conditional mutualism (Roossinck 2011; Carr 2017).

Recent years have also seen more studies on how viruses can influence insect vectors (reviewed in Mauck *et al*. 2012; Blanc *et al*. 2014; Fereres & Raccah 2015). However, the effects of plant pathogens on beneficial insects, including pollinators, has been little studied. Groen *et al*. (2016) and Jiang (2017) have revealed that CMV affects the interaction of tomato with buff-tailed bumblebees (see Section 1.5). Tomato flowers can self-pollinate, but this is inefficient without bumblebee-mediated “buzz pollination.” Buzzing maximizes the number of seeds produced per fruit. The bees also carry pollen between plants, facilitating cross-pollination. Groen *et al*. (2016) suggested that by making infected, susceptible plants attractive, the virus was ‘paying back’ susceptible hosts. This might increase the dissemination of its pollen, and its yield of seed and so may compensate for decreased yield of seeds on the virus-infected plants. This might inhibit natural selection for resistance (Groen *et al*. 2016).

1.7 Virulence in plant viruses

In ecological terms, if viruses have a restricted niche of one or few hosts, they can evolve to become specialists (Fry 1996; Kaltz & Shykoff 1998). Inversely, if viruses are able to infect and transmit from a wide range of hosts, they evolve to become generalists (Woolhouse *et al*. 2001). It has been shown that on average, specialist viruses are more virulent than generalists ones

(Agudelo-Romero & Elena 2008). Virulence of a virus has been defined as the relative amount of damage caused to a host (Shaner *et al*. 1992). Thus, the deleterious effects of virus infection on the host fitness that can either be severe symptoms or mortality.

Among the viruses used in this study, potyviruses BCMV and BCMNV are specialised legume infecting viruses (Section 1.4.1.2), whereas CMV in a generalist virus with one of the largest host range among all known viruses (Section 1.4.1.2). BCMNV has a less extensive distribution than BCMV, which has a worldwide distribution. For that reason, it is thought that BCMNV evolved from BCMV more recently (Spence & Walkey 1995; Gibbs 2008) (Section 1.4.1.2). Emerging viruses are usually more virulent and cause greater yield losses in crop production (Vuro *et al*. 2010). BCMNV, such as the isolate used in this study, causes severe symptoms and mortality in legumes as compared to BCMV (Section 1.4.1.2).

1.8 Hypotheses, aim and objectives

Plant viruses in agricultural ecosystems can provide model study systems for host-virus coevolution, speciation processes, and population dynamics (Stukenbrock & McDonald 2008). This is because selection pressures acting on hosts and plant viruses are interdependent, coupled and evolutionary changes may occur faster in these agricultural ecosystems compared to natural ecosystems (McDonald & Linde 2002; Zhan *et al*. 2002). Similarly, plant-pathogen and plant-pest interactions can drive co-evolutionary selection that produces genetic differences within populations (Laine 2009; Krings *et al*. 2012). Previous studies on co-evolution have focused on direct two-way plant-pathogen, plant-insect and insect-pathogen interactions (Begon *et al*. 2005). Recent investigations on three-way interactions of pathogens, insect vectors and host plants have shown that pathogens like viruses have intricate ways to ‘manipulate’ host plant defence and metabolism to alter insect vector behaviour in ways that may promote transmission (reviewed in Biere & Tack 2013). There is very limited literature on the effects of plant viruses on plant-pollinator

relationships, or on the evolutionary implications of viral modification on plant-pollinator interactions (Groen *et al*. 2016; Jiang 2017).

Experiments conducted in our laboratory by Groen (2013) demonstrated that VOC blends emitted by CMV-infected tomato and Arabidopsis plants altered the foraging behaviour of bumblebees (Groen 2013). When the same experiment was repeated with tomato plants infected with a mutant CMVΔ2b unable to express the 2b protein, bumblebees lost their innate preference for virus-infected plants (Groen 2013). Experiments with CMVΔ2b mutant in tomato and the inability of bumblebees to effectively distinguish between VOC blends emitted by *ago1* and *dcl1* Arabidopsis mutants implicated small RNA networks in regulating the emission of pollinator-perceivable volatiles (Groen *et al*. 2016). Greenhouse pollination experiments with mock-inoculated and CMV-infected tomato plants showed that bumblebee pollination in CMV-infected plants rescued seed production (Groen *et al*. 2016). CMV-infected plants that were not visited by bees produced significantly fewer seeds than mock-inoculated plants not visited by bees. When bees visited CMV-infected plants, seed production was restored to match up production in mock-inoculated plants (Groen *et al*. 2016). Using a mathematical model, Groen and colleagues (2016) predicted that in a hypothetical wild plant population an increased pollination bias by bees towards virus-infected would increase the reproductive success of infected plants. This will increase the passaging of alleles of virus susceptibility in progenies and hence favour the predominance of virus susceptible plants (Groen *et al*. 2016). In their conclusion, Groen and colleagues (2016) suggested that it might be a virus' interest to 'pay-back' a susceptible host by increasing the dissemination of its pollen by pollinators and might provide mutual benefits to the virus and its susceptible hosts.

It was against this background that my project was formulated, and my work was an extension of previous work by Groen (2013) and colleagues (Groen *et al*. 2016; Jiang 2017) who proposed a

payback hypothesis. The over-arching aim of my project was to investigate if the innate preference of bumblebees for virus-infected plants is unique or not to the tomato-CMV pathosystem. I sought to investigate if virus infection affected pollination behaviour of bees, the reproductive success of *P. vulgaris,* and if bees gained any improved benefits from pollinating virus-infected plants.

I hypothesise that viruses pay back plant hosts by attracting pollinators and the pollinators also receive a form of payback from the three-way interaction with possible long-term consequences for virus-host co-evolution. I tested this hypothesis in a different host plant to determine if it is tenable for plants outside the Solanaceae with a more complex flower, different pollination system, and different reward to pollinators. The morphology of bean flowers is different from tomato flowers, and the pollination techniques used by their bee pollinators are different as well (see section 1.3.1); tripping pollination in beans versus buzz pollination in tomatoes. Tomato flowers reward bee pollinators with excess pollen while bean flowers offer nectar rewards. I sought to further test the payback hypothesis not only with the model pollinator *B. terrestris* under controlled conditions but also in more natural environments (University of Cambridge Botanic Garden) where other wild bee pollinators are existent.

Objectives of my work were:

1. To determine the effects of BCMV, BCMNV and CMV viruses on *P. vulgaris* floral traits.
2. To determine if BCMV, BCMNV and CMV infection induces quantitative and qualitative changes in the blend of VOCs emitted by *P. vulgaris*.
3. To test if changes in olfactory cues caused by virus infection (BCMV, BCMNV and CMV) could be perceived by buff-tailed bumblebees in *P. vulgaris*.
4. To investigate if bumblebee-bean interactions are affected by virus infections (BCMV, BCMNV and CMV) and in turn, the reproductive success of *P. vulgaris*.
5. To investigate if bee-mediated pollination of virus-infected *P. vulgaris* affects the rate of virus seed transmission.

Objectives 1 and 5 were not previously done by Groen *et al*. 2016, hence new work. Objectives 2, 3 and 4 were similar to experiments by Groen *et al*. 2016, but in a new phyto- and pathosystem.

CHAPTER 2

MATERIALS AND METHODS

2.1 Laboratory reagents and non-biological material

2.1.1 Chemicals, molecular biology reagents and equipment

Most laboratory reagents and chemicals were purchased from Sigma-Aldrich (Gillingham, UK) or Fisher Scientific (Loughborough, UK). DNA markers (1 KB Ladders), loading dye and PCR premixes were obtained from Bioline Reagents Ltd. (London, UK). RNA markers were supplied by Promega (Chilworth, Southampton, UK). Benchmark pre-stained protein markers (6.0-181.8 kDa) and RNaseOUT® RNase inhibitor were supplied by Invitrogen (Paisley, UK). Other manufacturers of specific equipment or molecular biology reagents are indicated in the text. All solutions and reagents were prepared with autoclaved deionised, ultra-pure water ('Milli-Q' water) produced by a Millipore -Q Plus device (Millipore, Billerica, MA, USA).

2.1.2 Sterilization of equipment and solutions

Flasks, glass bottles, and plastic equipment were sterilized by autoclaving at 121°C for 15 minutes at 15 pounds per square inch pressure. Glassware and metal plates for headspace entrainment were soaked in 0.2% (v/v) Teepol (Teepol Products Ltd., Kent, UK) to remove organic residues, for a minimum of 30 minutes, followed by rinsing with 100% acetone to remove ionic residues, then deionised water and baked at 180 °C for 2 hours to degrade any remaining volatiles before use. Other glassware, ceramics and metal were soaked in 2.5% (w/v) sodium hypochlorite for a minimum of 30 minutes, washed in deionised water and baked at 180°C for 2 hours. Porapak Q tubes for volatile collection [containing filters of 50 mg packed spherical beads, 60/80 mesh size, Supelco (Sigma-Aldrich)] were conditioned by washing with diethyl ether and baked in an oven at 132°C for 2 hours before use. All solutions and media were prepared using sterile, ultra-pure MIlli-Q water and autoclaved. Single-use plastic 50 ml and 15 ml conical-ended centrifuge tubes

('Falcons'), 1.5 ml and 0.5 ml microfuge tubes, Petri dishes, "Phytotrays" and syringes were provided sterile and ready to use by BD Biosciences (Oxford, UK).

2.2 Experimental organisms

2.2.1 Plant material

Phaseolus vulgaris cv. 'Wairimu' / Red Haricot-GLP 585 (Simlaw Seeds, Nairobi, Kenya) and cv. 'Dubbele witte' ('Stamslaboon Dubbele witte') (van Hemert & Co, Tuinzaden.eu, Amsterdam, Netherlands) were used. Wairimu and Dubbele witte are susceptible to BCMV, BCMNV, and CMV and thus suited to my work. The architecture of cv. Dubbele witte is similar to cv. Wairimu with major differences in flower colour and seed number per pod. Dubbele witte flowers are white, and upon maturity, pods contain up to 8 seeds. Wairimu produces purple coloured flowers, and mature pods contain up to 5 seeds. The plant architecture is bushy and short. On-set of flowering takes approximately 45 days after planting. Inbred collections of *Vicia faba* L. (faba bean) cv. 'Fuego' NV641 and *Vicia faba* cv. 'Tatoo' NV676 (National Institute of Agricultural Biology: NIAB) were also used in initial experiments.

2.2.1.2 Planting and growth conditions

Plants for floral trait analyses and flight arena experiments with bumblebees were grown at the Plant Growth Facility (PGF), University of Cambridge Botanic Garden. Faba bean and common bean cv. Dubbele witte seeds needed surface sterilization prior to imbibition to prevent microbial infection whereas cv. Wairimu did not require pre-treatment because the supplier coats the seeds with chemicals for protection from microbes. Faba bean and common bean cv. Dubbele witte seeds were surface sterilized with 5% (w/v) sodium hypochlorite for 3 minutes and thoroughly rinsed with sterile water. Petri dishes (90 mm diameter) were used for imbibing the seeds. These had two sheets of 90 mm Whatman® filter paper (Whatman International Ltd, Maidstone, England)

placed in them, moistened with 6 ml of sterile water. Batches of five bean seeds were placed on the moist filter paper, and the Petri dishes were covered with their lids and sealed with Parafilm M® (Bemis Company, Inc, Neenah, USA) and incubated at 23°C for four days resulting in nearly 100% germination. The germinated seeds were planted out into disposable 23 cm X 12 cm rectangular pots on the fifth day of imbibition (one seed per pot) filled with 4:1 Levington M3 compost (Fisons Plc., Ipswich, UK): sharp sand mixture [washed, lime-free, horticultural quartzite sharp sand: (J Arthur Bowers, Lincoln, UK)]. They were labelled accordingly (species name and date of planting) and placed in a custom-built walk-in growth room (Conviron, Manitoba, Canada) at the PGF on automatic irrigation. Growth conditions were set at 20-22°C, 60% relative humidity under short photoperiod (8 hours light and 16 hours darkness) and 200 µmol/m²/s photosynthetically active radiation (using Sylvania Activa 172 Professional 36W bulbs).

Plants for pollination experiments were grown in the University of Cambridge Botanic Garden glasshouse. The growth conditions were maintained at 15–25°C, Lucalux LU 400 W/PSL lights were automatically activated between 4 am and 8 pm when light levels fell below 150 W/m². Humidity was approximately 55%. Plants were randomly placed within the glasshouse space available to minimize variation in humidity or light.

Western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) were a common pest in the PGF and glasshouse. They were controlled by the predatory mite *Amblyseius andersoni* Chant (Acari: Phytoseiidae) (Syngenta® Bioline® Anderline™) and Pheromone lures (Thripline $_{ams}$, Syngenta Bioline Ltd, Clacton, UK), each containing 30 µg of the *F. occidentalis* aggregation pheromone, neryl (*S*)-2-methylbutanoate. Two-spotted spider mites (*Tetranychus urticae* Koch) (Acari: Tetranychidae) were problematic in the glasshouse. They were controlled by predatory mites *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) (Bioline AgroSciences Ltd., Essex, UK) and *A. andersoni* (Syngenta® Bioline® Anderline™).

Plants for outdoor pollination experiments in the University of Cambridge Botanic Garden experimental plots were raised in the glasshouses before transplanting at 5 days post-inoculation in the garden (see Section 2.7). Some of the plants were also grown in the glasshouse in pots and were translocated to the Garden upon flowering (Section 2.7). These experiments were done during summer from mid-May to mid-September of 2017 and 2018.

2.2.2 Viruses

European isolates of the viruses used in this study were from the Leibniz-Institut DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH: German Collection of Microorganisms and Cell Cultures) as freeze-dried leaf material. These were BCMV isolate PV- 0915, BCMNV isolate PV-0413 and CMV isolated from bean (isolate PV-0473). CMV PV-0473 has a similar RNA sequence to a bean-infecting isolate from common bean from western New York State (isolate Bn57), described by Thompson *et al*. (2015). Viruses were passaged in *P. vulgaris* by sap inoculation (see Section 2.3). Sap inoculum was initially prepared from 50 mg of freeze-dried leaf from the stock centre that was ground using a mortar and pestle in sterile nuclease-free water.

2.2.3 Bumblebees

Bombus terrestris audax (Koppert, Berkel en Roderijs, The Netherlands and Syngenta-Bioline, Leicester, UK) were supplied in boxed colonies and connected to a 72 x 104 x 30 cm wooden flight arena by a transparent plastic tube (modified from Whitney *et al*. 2008) (Figure 2.1). The flight arena floor was painted green with “Garden Green” water-based enamel paint (Plasti-kote, Wokingham, UK) as a neutral background and had a clear ultraviolet (UV)-transparent Plexiglass lid. Bumblebees were free to forage in the arena, but during experiments, their movement was restricted using a series of manually controlled gates. They were fed daily with 30% (w/v) sucrose solution placed in 0.2 ml plastic feeding rack tubes. The colony was fed with pollen thrice a week by opening an in-box feeding system. The flight arena was illuminated by six Sylvania Activa 172

Professional 36 W fluorescent tubes that were modified to a flicker frequency > 1,200 Hz (Figure 2.1).

2.3 Virus inoculation

All experiments required bean plants to be infected with BCMV, BCMNV and CMV. A batch of mock-inoculated plants was also essential as controls. All plants were inoculated when the first two true leaves had emerged. For common bean, this was after four days from planting out and nine days for the two faba beans varieties. Mechanical inoculation was used whereby the true leaves were dusted with Carborundum [silicon carbide (SiC)], and sap inoculum rubbed onto the leaves using fingertips with latex gloves on. Carborundum ensured abrasion for virus entry into the leaves. The inoculations were also done for bulking up of viruses on common bean cv. Wairimu and thus subsequent sap inoculum preparations were done using freshly harvested virus-infected leaves of these plants. Mock inoculation was done using the same procedure as virus inoculations using sterile water. An adjustment to the inoculum for mock treatment would have been the use of sap from a healthy plant in place of sterile water so as to exclude for other factors that may have been introduced via sap (Dr Betty Chung, personal communication). Inoculated plants were grown the PGF (from henceforth referred to as controlled conditions) (Section 2.2.1). Systemic infection was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) assays (Section 2.4).

2.4 Confirmation of systemic infection of bean plants

Symptoms of virus infection on common bean and faba bean hosts were recorded and photographed. Plants were randomly selected, and fresh leaf samples were collected, wrapped in labelled foil, immediately flash-frozen in liquid nitrogen, and sent to the laboratory using a dry shipper for further processing. Samples were stored at -80 °C for subsequent processing.

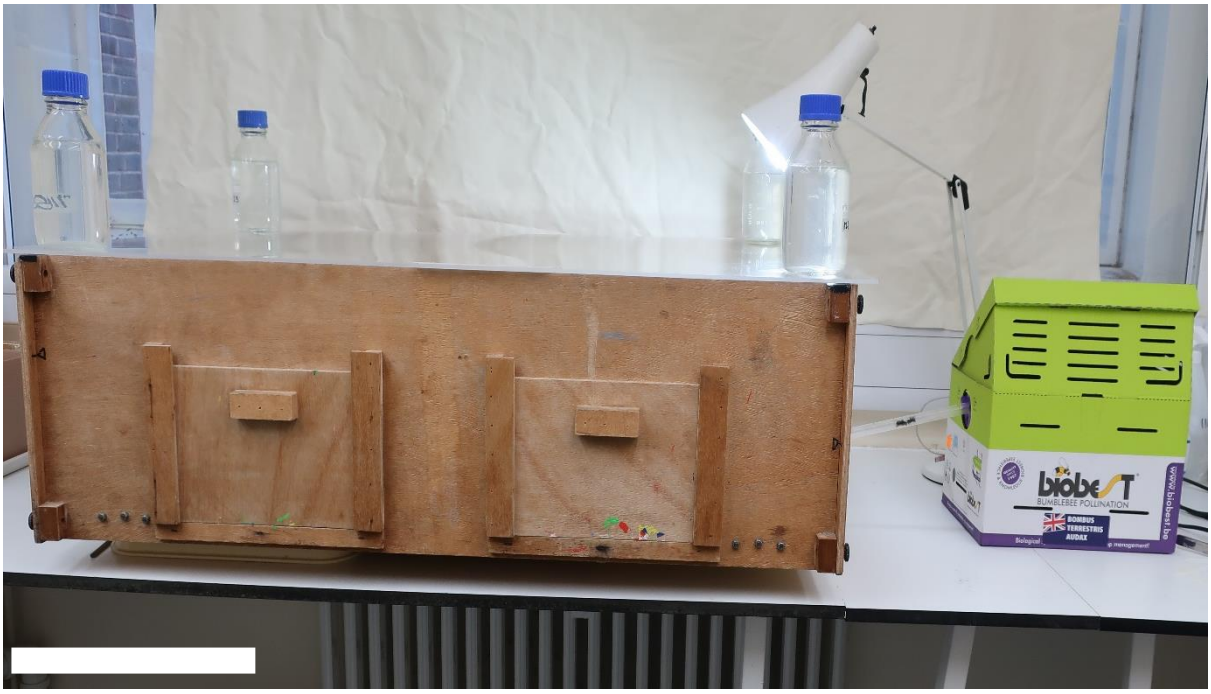


Figure 2.1 Flight arena. The wooden arena connected to a colony of *B. terrestris audax* in a cardboard box by use of a gated plastic tube. Bumblebees are free to forage in the wooden arena. During experiments, the movement of bumblebees into the arena was controlled by opening and closing gates along the plastic tube. See Figure 2.6, which shows the internal arrangement of the arena. Scale bar = 30 cm.

2.4.1 RNA extraction

The procedure used to extract RNA was adapted from Wamonje (2017). A 1 cm diameter cork borer was used to obtain approximately 50 mg samples of leaf tissue (section 2.4) and put into sterile 1.5 ml microfuge tubes containing 500 µl 'TRIzol-like' extraction buffer [38% v/v Tris-buffered phenol. 0.8 M guanidine thiocyanate, 0.1 M sodium acetate pH 5.0, 5% v/v glycerol]. Solutions used for RNA extraction were made in sterile water. Leaf samples were homogenised using a micro pestle and placed on ice for 5 minutes to allow complete dissociation of nucleoprotein complexes and then centrifuged at 10 000 xg for 10 minutes at 4°C in a precooled HERMLE® Z-400 K benchtop centrifuge (HERMLE Labortechnik, Wehingen, Germany). Supernatants were transferred into sterile microfuge tubes, and 100 µl of chloroform: isoamyl alcohol (24:1) added to the supernatants and vortexed for 30 seconds to sequester phenol. Samples were centrifuged at 10,000 xg for 15 minutes in the pre-cooled desktop centrifuge. The upper (aqueous) phase containing RNA (approximately 300µl) was transferred from each sample into new microfuge tubes and equal volumes (300 µl) of isopropanol pre-cooled to -20°C was added. After briefly vortexing, samples were placed at -20°C for 2 hours to precipitate nucleic acids. The mixtures were centrifuged at 10,000 xg for 15 minutes, and the RNA precipitates formed gel-like pellets either at the bottom or on the side of the tubes hence the supernatant was poured off. Pellets were washed once by mixing with 300 µl of 70% ethanol (v/v) and centrifuging for 5 minutes at 10 000 xg. The ethanol was poured off and the pellets air-dried before being dissolved in 400 µl nuclease-free water.

2.4.2 Quantitative analysis of RNA concentration and purity

Extracted RNA was analysed for concentration and purity using a Nanodrop® ND1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Sterile nuclease-free water was used as a blank, and 1 µl of the extracted RNA from each sample was used in the quantification. The UV absorbance of RNA samples at 280 nm and 260 nm was determined, with

R260/280 of 1.8-2.0 considered indicative of high purity standards, whereas lower ratios would be indicative of protein contamination.

2.4.3 Reverse transcription and PCR

Procedures used were adapted from Wamonje (2017). RNA was reverse-transcribed using the GoScript™ (Promega) reverse transcription system. Total plant RNA (up to 500 ng) in 1µl was mixed with 1 µl (0.5 µg/µl) of oligo $(dT)_{15}$ (Sigma) for BCMV and BCMNV samples and random primers for CMV samples and topped up to 5 µl with nuclease-free water, incubated at 70°C for 5 minutes, and placed on ice to anneal the primers. The GoScript reverse transcription mix was prepared according to the manufacturer's instructions [4 µl GoScript 5X reaction buffer, 3 µl $MgCl_2$ (final concentration 2.4 mM), 1 µl 10 mM dNTP mix, 1 µl ribonuclease inhibitor (final conc. 1.33 U/µl), 1 µl GoScript reverse transcriptase enzyme (final conc. 0.333 U/µl) were mixed and topped up to 15 µl with nuclease-free water] and mixed with 5 µl of RNA and primer mix made in the initial step. The mix was annealed at 25°C for 5 minutes, extended at 42°C for 60 minutes and then the reverse transcription enzyme was inactivated by incubating at 70°C for 15 minutes. Tubes containing cDNA were immediately placed on ice for use in the PCR using specific primers.

PCR was carried out using 2X Biomix Red® premix according to the manufacturer's instructions [contains: BIOTAQ DNA Polymerase (5 U/µl), 2 mM dNTPs, 32 mM $(NH4)_2SO4$, 125 mM Tris-HCl (pH 8.8 at 25°C), 0.02% Tween 20, 3 mM $MgCl_2$ Stabiliser, Innate Dye] (Bioline Reagents Ltd.). Five µl of the premix was mixed with 3.6 µl of nuclease-free water, 1 µl of the cDNA template (50 ng/µl) and 0.4 µl each of specific coat protein forward and reverse primers for each of the virus tested. PCR cycling conditions were initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, an annealing step at 54°C, an extension step at 72°C for 1 minute and a final extension of 72°C degrees for 10 minutes in a thermal cycler (ABI-Applied Biosystems, Veriti, USA).

2.4.4 Gel electrophoresis of DNA

PCR products were loaded into wells of a 1% (w/v) agarose gel in TAE (Tris-Acid EDTA) buffer [0.04 M Tris, 1mM EDTA pH 8.0, 0.1142 % (v/v) glacial acetic acid] containing 0.05 $\mu g.ml^{-1}$ ethidium bromide. A 1.5 Kb ladder (Bioline, London, UK) was also loaded to facilitate PCR product size estimation. The gels were submerged in TAE buffer and run in an MHU–1010 gel rig (Flowgen/Scientific Laboratory supplies, Hessle, UK) at 100V using a Power-Pac 3000 (Bio-Rad, Hemel Hempstead, UK). Gels were examined with UV illumination on an AlphaImager® gel documentation system (AlphaInnotech/ ProteinSimple, Santa Clara, CA, USA).

2.4.5 Seed transmission screening using the Enzyme-linked immunosorbent assay

Progeny of infected bean plants from glasshouse and Garden pollination experiments (bumblebee -pollinated or self-pollinated) and controlled conditions were tested for seed-borne virus transmission by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) as described in Gan & Patel (2013). BCMV, BCMNV and CMV ELISA complete kits (Bioreba AG, Reinach, Switzerland) were used to detect coat proteins of these viruses. Bean seeds were imbibed and germinated as described in Section 2.2.1. In each test, the virus-specific antibody (anti-BCMV IgG; anti-BCMNV IgG; anti-CMV IgG) was diluted 1000 x in coating buffer, and 200 µl was added to each well on a 96-well plate (Immuno Plate F96 MaxiSorp, Scientific Laboratory Supplies, East Riding, Yorkshire, UK). The plate was covered with Saran Wrap (Dow Chemical), placed in a humidity box overnight at 4°C. On the second day, about 0.05g of the shoot plumule samples were harvested from the developing shoots (Figure 2.2) and homogenized 1:20 in (137 mM w/v NaCl mM, 3 mM w/v KCl, 1 mM w/v $MgCl_2$, 2% PVP 24 kD, 0.05% v/v Tween-20, 0.2% BSA, and 0.02% NaN_3, 20 mM, pH 7.4). The samples were centrifuged at 13,000 rpm for two minutes and kept on ice. Wells were washed three times using phosphate-buffered saline (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween-20, pH 7.4.) and 200

µl of each sample added per well. The plate was covered and placed in a humidity box overnight at 4°C. On the third day, the wells were washed three times with phosphate-buffered saline. The second antibody, (anti-BCMV CP) IgG; anti-BCMNV CP IgG; and anti-CMV CP IgG) conjugated to alkaline phosphatase, was diluted 1000 x in 137 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2% PVP 24 kDa, 0.05% v/v Tween-20, 0.2% w/v BSA, and 0.02% NaN3, 20 mM Tris buffer, pH 7.4), and 200 µl was added to each well. The plate was covered and incubated at 28°C for four hours and then washed three times. 200 µl of *para*-nitrophenyl phosphate at 1 mg/ml, dissolved in substrate buffer [1 M diethanolamine (pH 9.8), 0.02% w/v NaN_3], was added to each well. Samples were incubated at room temperature for 30 minutes to allow a yellow colour change to develop and this was measured using the plate reader spectrophotometrically at 405 nm against buffer as a blank (Titertek Mutliskan Plus) with DeltaSoft software.

2.5 Examination of flower development and floral traits

Virus-infected and mock-inoculated *P. vulgaris* cv. Wairimu plants were allowed to grow until flowering. Records were kept of stages, that is: date seeds were sown; inoculation date, when plants produced their first floral buds, and when the first flowers fully opened. A summary of floral traits that were measured in the current study is summarised in Table 2.1.

Legume flowers mature from buds to open flowers. The standard petal moves from tightly enclosing the bud to reflexing back away from the keel-wing complex until eventually the sides of the standard petal touch. In old flowers, the standard petal then collapses back to enclose the keel-wing complex containing the carpel (Osborne *et al*. 1997). During this project, fully open flowers (Figure 2.3A) were predominantly studied, as this stage has been shown to be when legume flowers are most likely to be visited by bees (Pierre *et al*. 1996).

2.5.1 Measurements of flower morphology

Fresh open flowers (Figure 2.3) were collected and immediately photographed in their natural conformation so that the measurements taken reflected the trait values that a bee visiting a flower would encounter in the field. A Canon EOS KissX4 DSLR or Canon EOS 20D DSLR camera equipped with Canon EFS 18-55mm lens was used. Photographs were captured of the face and side view of fresh fully open flowers against a black background at PGF (Figure 2.3B). A PCR tube was used to support the calyx to ensure that flowers were parallel to the background without altering the floral morphology. This was done to prevent measurement errors from photographs being taken at different angles. Each flower was dissected, and floral components were photographed together. All images contained a scale bar so that the measurements taken from the images could be scaled appropriately. Side view images were straightened to be parallel to the top of the calyx so that consistent measurements could be made. The images were then analysed using ImageJ (http://rsb.info.nih.gov/ij/), calibrated to a scale bar in each photograph, using the point and polygon tools. All the floral traits were measured from flowers in their natural conformation so that they reflected the trait as a pollinator would perceive it. Measurements taken are shown in Figure 2.3B (the exact location of the measurements). These were flower length, corolla tube length and area of the wing and standard petals as observable by bees in the environment (Figure 2.3B). Thirty replicates from each treatment (3 replicates per plant; 10 plants) were used for these measurements.

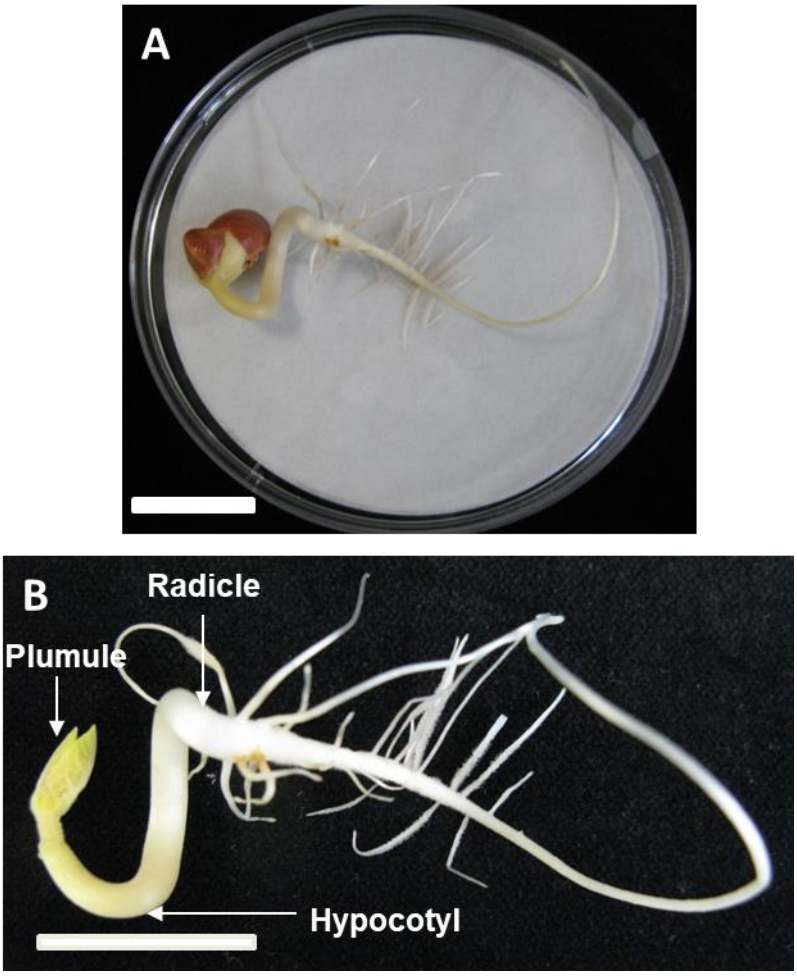


Figure 2.2 Seedlings used in virus seed-borne transmission screening. (A) Seedling on petri-dish after incubation. (B) Seedling showing plumule after removing seed coat and cotyledons. The plumule was harvested for screening of presence of virus. Scale bars = 2 cm.

Table 2.1 Floral traits that were analysed

Category	Floral traits that were measured
Flower morphology	Flower length
	Corolla tube length
	Standard petal area
	Wing petal area
Flower colour and patterning	Standard petal venation colour
	Wing petal colour
Epidermal cell morphology	Conical cell distribution
VOC	VOC produced
Reward	Nectar volume
	Nectar sucrose concentration

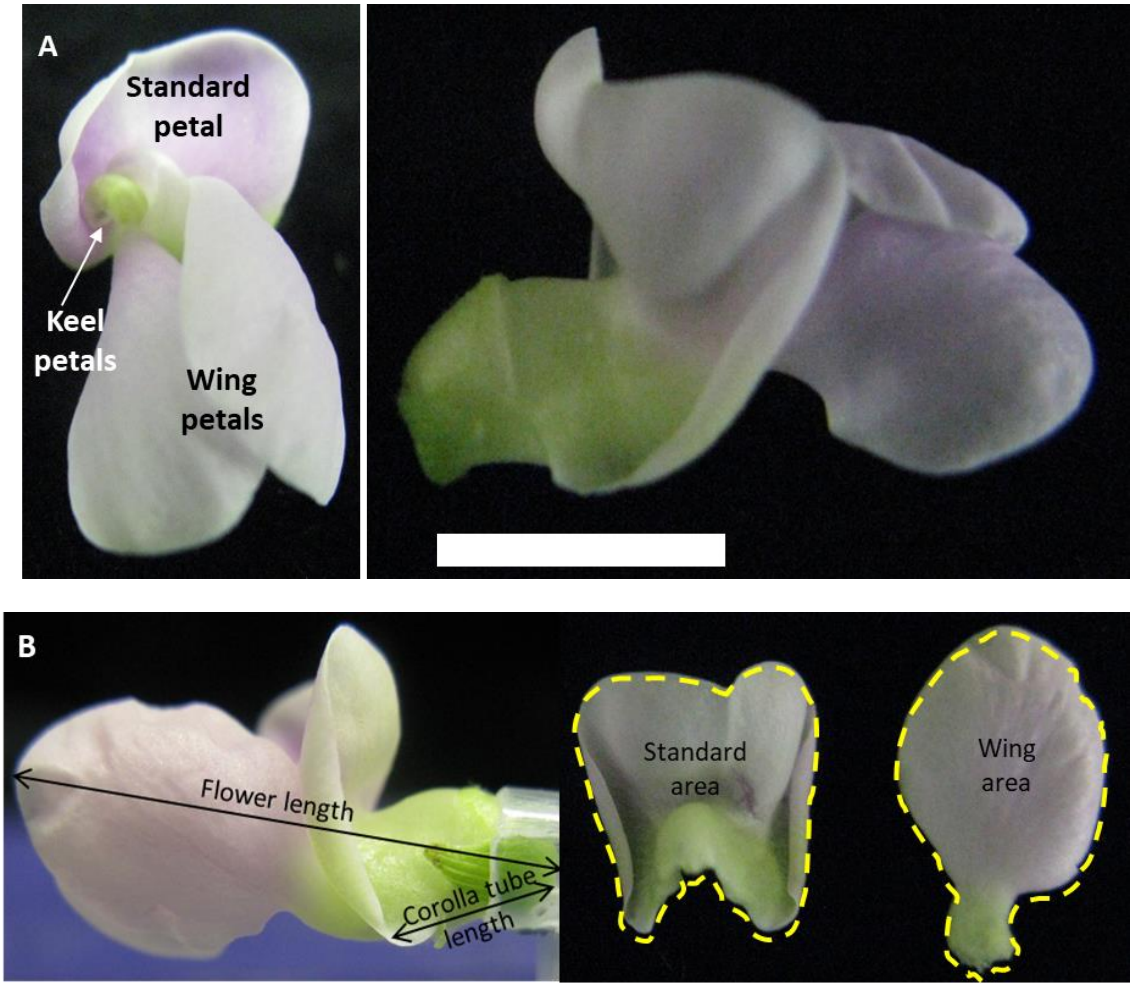


Figure 2.3 Fully open flower of cv. Wairimu

This is the stage at which bees are most likely to visit *P. vulgaris* flowers. Panel A shows left: face view and right: side view of the open fully flower. Standard and both wing petals are erect and standard petal is reflexed. All floral trait measurements were taken at this stage of fully opened flowers. Scale bar = 1 cm. Panel B illustrates the floral measurements taken. Left: flower length was measured from tip of calyx to tip of wing petal and corolla tube length measured from tip of calyx to tip of standard fold. Flowers were held into position in their natural conformation by use of PCR tubes. Right: Area of standard and wing petals in their natural conformation as perceived by bees.

2.5.2 Measurement of floral colour as perceived by bees

Procedures by Bailes (2016) were followed to measure flower colour. Fully open flowers were collected from each treatment and transported to the Department of Plant Science in 50 ml centrifuge tubes containing moist tissue paper to prevent wilting. The reflectivity of two points of interest was measured because they were identified as those most likely to be seen by a pollinator approaching a flower and after landing in search for nectar. These points of interest were: (i) tip of the abaxial face of the wing and (ii) venation of adaxial face of the standard petal. Floral petals were carefully flattened onto a glass microscope slide using double-sided sticky tape to reduce experimental error from light scattering. Samples were placed in a black lined box to prevent light from external sources from affecting the amount of light reflected by the petal sample. The percentage reflectance of samples was measured and recorded using a spectrometer (Ocean Optics 2+), with a 10 ms integration time, and the reflectance spectrum of the black background was corrected. The samples were illuminated with a Deuterium-Halogen light source (Ocean Optics DH 2000) and analysed with SpectraSuite software (version 1.0, Ocean Optics). For each treatment, the reflectance of 15 flowers in each treatment, thus three flowers from five different plants was measured. The reflectance values at each wavelength per petal per treatment were then averaged. This average reflectance across a spectrum was then converted to a co-ordinate in bee colour-space following Chittka (1992) (Section 1.3). This allowed a quantitative comparison of the floral colour of treatments as recognisable by bees.

2.5.3 Examining epidermal cell morphology of wing petals

Three freshly open flowers were collected from three plants (one flower per plant) across all treatments, and dental wax (Zhermack Clinical, Italy) casts were made of the abaxial surface of wing and adaxial side of the standard petals. One cast of each petal was made. Epoxy-resin (ITW Devcon, USA) replicas were produced from these dental wax casts. The cell surface replicas were then mounted onto aluminium stubs and sputter-coated with silver or gold using a Quorum K756X

sputter coater. Coated samples were examined using an FEI Philips XL30 Scanning Electron Microscope (SEM) at the Multi-Imaging Centre of the University of Cambridge. Petals were surveyed for presence or absence of conical cells with reference to Christensen & Hansen (1998) and Ojeda *et al*. (2009). Cell types found on legume epidermis are Tabular Rugose Granular, Tabular Rugose Striate, Tabular Flat striate, Papillose Conical Striate, Papillose Knobby Rugose, and Papillose Lobular Striate (Figure 1.3) (Christensen & Hansen 1998; Ojeda *et al*. 2009). The distribution of these cell types was also noted, along with any other interesting epidermal features.

2.5.4 Measurement of nectar production

Methodology for nectar production measurements was adapted from Bailes *et al*. (2018). Nectar produced by fully opened flowers was measured from flowers harvested randomly between 10 am, and 12 am on plants which had been flowering for at least 1 week and for no more than 2 weeks. These requirements were made due to the high variability in the production of nectar both over the course of the day and over the lifetime of a plant (Kakutani *et al*. 1989; Osborne *et al*. 1997). Nectaries at the base of the reproductive complex were exposed by carefully removing petals and calyx. The reproductive complex was placed into a 0.5 ml microfuge tube with 3 holes pierced at the base (Figure 2.4). This tube was then placed in a pre-weighed 1.5 ml microfuge tube and centrifuged at 13,000 rpm for 2 minutes. The 1.5 ml microfuge tube was weighed, and the difference between the microfuge after and before centrifugation gave the mass of the nectar. Four flowers per plant were measured from 25 plants across all treatments.

Sucrose content (w/v) was quantified using a handheld refractometer (Eclipse 45-03, Bellingham and Stanley, Thermo Scientific, UK) which measures in 0.5 % divisions from 0 – 50 %. Only flowers which produced at least 1 µL of nectar could have their sucrose content measured because at least 1 µL of sample is required to give an accurate reading. Four flowers per plant were measured where possible from 25 plants per treatment. The volume of nectar extracted from

a flower was calculated using the mass of nectar weighed in mg (**M)** and the concentration of its nectar in % sugar w/w (**C**) using the equation below (Bailes *et al*. 2018):

$$Volume\ (\mu L) = \frac{\boldsymbol{M}}{0.9988603 + \ 0.0037291\mathbf{C} + 0.000178\boldsymbol{C^2}}$$

It was assumed that all sugar was sucrose, the predominant sugar contained in legumes, including *P. vulgaris* nectar, according to Pierre *et al*. (1996). But as the other sugars found in substantial quantities in nectar, glucose and fructose, form similar density solutions and have comparable molecular weights, there would therefore be negligible difference in the final volume calculated.

2.6 Volatile Organic Compound Analysis

2.6.1 Headspace entrainment

The volatile organic compounds (VOCs) were captured using headspace air entrainment from cv. Wairimu flowers, flowering cv. Wairimu plants and both non-flowering and flowering cv. Dubbele witte bean plants following procedure by Groen *et al*. (2016). Four dynamic headspace trappings were done from each treatment. For cv. Wairimu flowers, each collection was done using 8 open flowers (stage 4 to 5 as described by Osborne *et al*. 1997) from 3 – 4 plants of the same treatment that were placed a 10 ml Pyrex glass beaker filled with distilled water. Plants were not re-used between replicates. The flowers were sealed in a glass collecting vessel using a glass base plate secured by bulldog clips. In volatile collections from non-flowering and flowering plants, each bean plant was placed singly in a glass chamber (190 mm high x 100 mm wide) with the bottom sealed off using two semi-circular metal plates with a hole in the centre to accommodate the stem (Figure 2.5). Open space around the stem was sealed with PFTE tape (Gibbs & Dandy, Luton, UK).

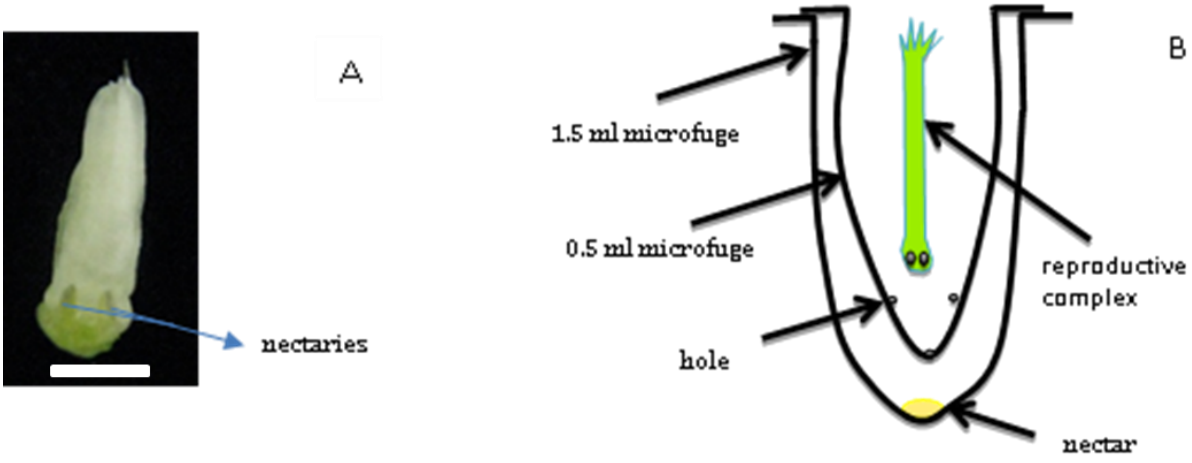


Figure 2.4 Extraction of nectar from nectaries of *P. vulgaris* by centrifugation. (A) The location of the nectaries in *P. vulgaris*, exposed by removing calyx and sepals from the reproductive structure of a flower. Scale bar = 1 mm. (B) Diagram illustrating microfuge tubes with reproductive complex and collected nectar after centrifugation at 13,000 rpm. Nectar collected at the bottom of the 1.5 ml microfuge tube during centrifugation through the three holes made on the 0.5 ml microfuge tube. A minimum of 48 flowers per treatment was used for nectar extractions. Diagram courtesy of Dr Emily Bailes (Bailes 2016).

Air was cleaned by passing through a charcoal-filter before entering the bottom of the chamber containing plants at a rate of 800 ml/min (Figure 2.5). Air was removed from the chamber at a rate of 700 ml min^{-1} through a Porapak Q filter contained inside a glass gas chromatograph (GC) inlet liner between two plugs of silanised glass wool, preventing contamination of the air from that outside the chamber (Figure 2.5). The Porapak Q filter trapped the volatiles from the samples. Headspace collection was carried out for 24 hours. The entrained VOCs were then eluted from the column using 700 µl of diethyl ether. Samples were stored at -80 °C. Fresh and dry weights of flowers were measured to enable normalization of the volatile abundance. The column was then washed as described in section 2.1.2. Leaf, leaf and flower, and flower fresh weight and dry weight were measured from non-flowering plants, flowering plants and cv. Wairimu flowers used in headspace entrainments respectively, to enable normalization of the volatile abundance. The VOC samples were analysed by coupled GC-mass spectrometry (GC-MS).

2.6.2 Coupled Gas Chromatography-Mass Spectrometry

Volatiles were separated on a capillary GC column (TG-SQC, 15 m by 0.25 mm; film thickness, Thermo Scientific, UK) for initial investigation of VOCs by principal component analysis (PCA) (Groen *et al*. 2016). The injection volume (splitless) was 1 µl, injector temperature was 200°C, and helium was used as the carrier gas at a constant flow rate of 2.6 $ml.min^{-1}$ in an oven maintained at 30°C for 5 minutes and then programmed to increase the temperature to 15°C/min to 230°C. Total run time per sample was 18.33 minutes. The column was directly coupled to a mass spectrometer (ISQ LT, Thermo Scientific, UK) with an MS transfer line temperature of 240°C. Ionization was by electron impact with an ion source temperature of 250°C in positive ionization. Mass ions were detected between 30 and 650 m/z. Data were collected using Xcalibur software (Thermo Scientific, UK). Principal component analysis of the mass spectra was performed with MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/) using binned m/z and percent total ion count (%TIC) values. Identities of VOCs in the blends emitted by the infected and mock-

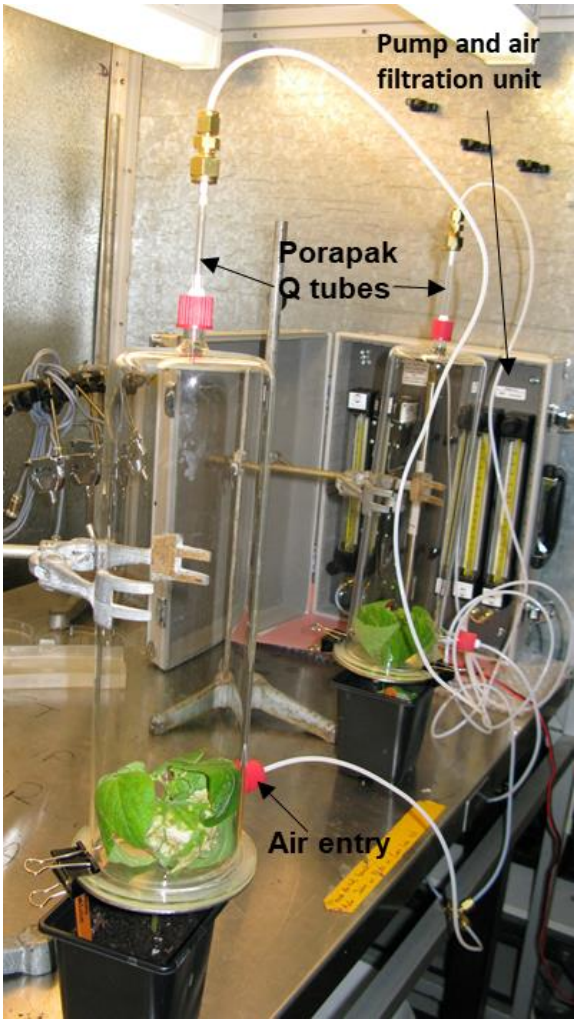


Figure 2.5 Experimental set-up for VOCs headspace entrainment.

Plants were placed individually in a 1-litre glass chamber, and VOCs were collected in the Porapak Q tubes inserted in the top. The bottom was sealed by two semicircular metal plates with a hole in the centre to accommodate plant stems. Charcoal-filtered air was pumped in at the bottom of the chamber at the rate of 800 ml/min, and drawn out through Porapak Q tube at the rate of 700 ml/min.

inoculated plants were confirmed by comparing the spectra with those in the National Institute of Standards and Technology (NIST) spectral databases (http://www.nist.gov). Plants used for VOC collection were not equally sized. Therefore, there was need to correct the quantitative data on VOC production by dry mass of the plant that yielded the respective sample (i.e., divided relative peak areas by the dry weight in grams).

2.7 Bumblebee Choice Assay tests

Free-choice assays were carried out to establish whether bumblebees were preferentially attracted to volatiles emitted by Spectrometry bean plants to mock-inoculated bean plants (Groen *et al*. 2016). Differential conditioning assays were done to determine whether bees could learn to associate volatiles with either a reward or punishment (Groen *et al*. 2016).

2.7.1 Training of bees

A week before carrying out conditioning or free choice assays, sucrose feeders were replaced by cylindrical feeding towers which would be used during experiments. This was done to familiarise and train bumblebees to forage from the towers with a reward. The towers were made from black card rolled into hollow cylinders (diameter 8 cm and height 15 cm) held in place with black masking tape (Figure 2.6A). Tower height was selected because bumblebees cannot effectively resolve visual cues beyond 10 cm (Chittka & Raine 2006). A muslin cloth (improvement for Groen *et al*. 2016 method) and then mesh roof were attached on the top using rubber bands. The muslin cloth aided in the elimination of visual cues because common bean plants used in experiments were almost as tall as the feeding towers, unlike in Groen *et al*. (2010) where tomato plants used were as shorter; their height was approximately half the height of feeding towers. The mesh supported a glued 1.5 microcentrifuge tube in which sucrose solution was repeatedly filled, acting as reward (Figure 2.6B). Actively foraging bees were marked on the thorax with water-soluble paint for use during assays.

2.7.2 Free choice assays

Free choice assays were done in the same way as in Groen *et al*. (2016). On the day of the experiment, all bumblebees were allowed to return to the colony box. The arena floor was wiped down with 30% (v/v) ethanol to remove any scent marks. Five mock-inoculated and five-virus infected bean plants were concealed under the towers and randomly placed in the arena (Figure 2.6A). This was done to eliminate visual cues and only allow volatiles emitted by the plants to be detected by bumblebees from the top of the towers. A drop (approximately 50 µl) of 30 % (w/v) of sucrose reward was placed in each micro-centrifuge tube to encourage bumblebees to feed from several towers before returning to the colony. Ten marked bees were then released one at a time and allowed to forage until satisfied. The first 10 choices of each bee tested for each pair-wise comparison was recorded either as an acceptance or rejection of choice. An acceptance was when the bee lands and feeds from the sucrose tower of a chosen plant (Fig 2.6B), and rejection was when the bee lands but does not feed from the sucrose tower. In-between feeding bouts, plastic meshes were cleaned with Q-tip cotton wool bud dipped in 30% (v/v) ethanol to remove scent marks and tube lids were refilled with sucrose. The towers were randomly re-arranged to avoid spatial learning by the bees. These experiments were done with non-flowering and flowering common bean plants infected with BCMV, BCMNV, CMV and mock-inoculated ones. Each bee was used once and removed from the flight arena after making its first 10 choices.

2.7.3 Differential Conditioning Assay

Differential conditioning assays methodology was adapted from Groen *et al*. (2016). The same experimental setup was used as described in Section 2.7.2, with differences as follows. Five flowering mock-inoculated plants were covered individually by towers with sucrose solution (30% w/v) as a reward, and five flowering BCMNV-infected plants were covered individually by towers with quinine hemisulphate solution (0.12% w/v) as punishment. Bumblebees are not able to distinguish between quinine hemisulphate solution and sucrose solutions based on any other cues

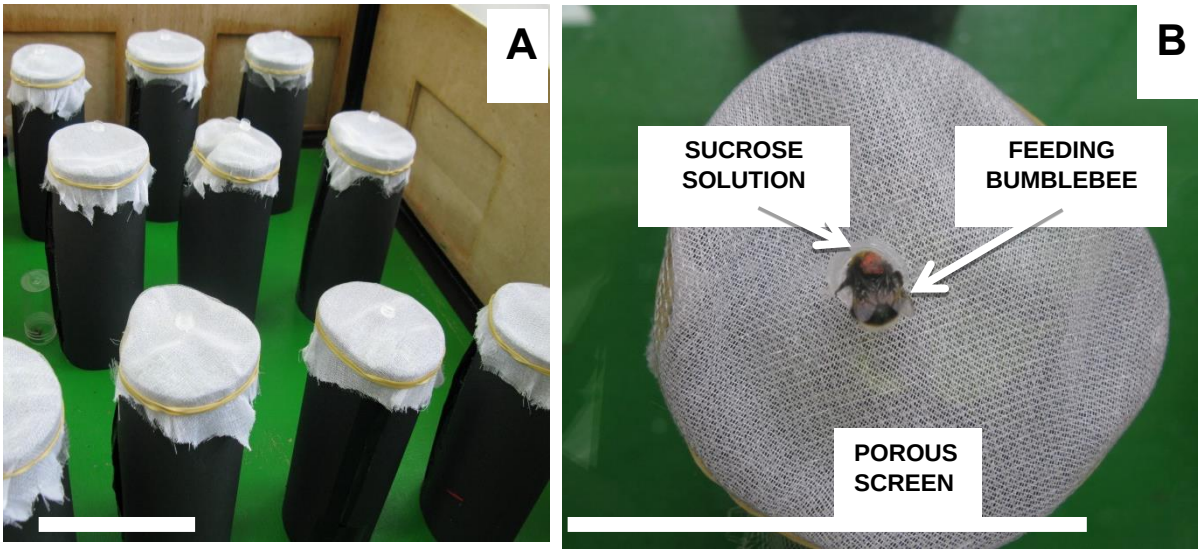


Figure 2.6 Experimental set-up for bumblebee choice assay tests.

(A) Feeding towers arranged in the wooden arena with five mock-inoculated and five virus-infected *P. vulgaris* plants hidden underneath them. The towers serve to eliminate visual cues and only allow VOCs to be detected by bumblebees from the top. (B) A marked bumblebee feeding from a feeding tower, this behaviour was considered as an acceptance. Scale bars = 8.6 cm

other than gustatory cues (Whitney *et al.* 2008). The first 100 choices of each 8-10 bees tested were recorded. A 'correct' choice was recorded when a forager drank on towers offering sucrose or aborted landing or hovered over towers offering quinine.

2.7.4 Learning Curve Data Analyses

The experimental protocol recorded the number of 'correct' choices made by each bee, and these were grouped into sets of 10 successive choices for ease of scoring. The learning curves data were analysed using binomial logistic regression. Exploratory analyses suggested no pronounced differences between individual bees within treatment groups, and so the data was fitted into a fixed-effect model (Eqn I)

Eqn I

$$b_{ij} \sim \mathrm{Bin}(10, p_i),$$
$$\log\left(\frac{p_i}{1-p_i}\right) = \alpha_0 + \alpha_1(i-0.5)$$

where b_{ij} is the number of correct choices made by the j^{th} bee in its i^{th} set of ten choices, p_i is the probability of choosing correctly in each successive batch of ten choices, and where α_0 and α_1 are the parameters to be estimated. Hosmer-Lemeshow tests were used to assess model goodness-of-fit: in all cases, there was no evidence for lack-of-fit. The parameter α_1 was hence assessed whether it was different to zero via a likelihood ratio test against the simpler nested model with α_1 fixed to be zero. Since the parameter α_1 controls how the (logit) of the probability of making a correct choice p_i increases with i, positive values of α_1 correspond to the bees 'learning' over time. Any systematic differences in the rate at which bees learn between pairs of experiments were assessed by simultaneously fitting a single regression model to the results of both experiments, allowing the probabilities of making a correct choice to depend on the experiment (Eqn II) in which E is an indicator variable which is equal to zero for the first experiment, and equal to one in the

Eqn II

$$\log\left(\frac{p_i(E)}{1-p_i(E)}\right) = \alpha_0 + (\alpha_1 + \alpha_2 E)(i - 0.5)$$

second experiment. A value $\alpha_2 \neq 0$ corresponds to bees learning at a different rate in the different experiments: again, this was tested via a likelihood ratio test against the simpler nested model in which α_2 was fixed to be zero. These equations for statistical analysis were provided by Dr Nik Cunniffe (University of Cambridge, Cambridge, UK) and previously published in Groen *et al*. (2016).

2.8 Glasshouse pollination experiments

These experiments were done in the University of Cambridge Botanic Garden glasshouse following basic set up by Groen *et al*. (2016). During experiments, a bumblebee colony was introduced into a large flight arena (320 x 440 x 210 cm, H x W x D) constructed from nylon netting (JoTech-Insectopia Ltd., Austrey, UK) (Figure 2.7A). The bumblebees were free to come in or out of the colony box, and trip pollinate flowers on six mock-inoculated and six virus-infected common bean plants that were arranged in alternating order of four plants in a row (Figure 2.7B). All flowers were left accessible to the bees (Figure 2.7C). Two people were present at either end of the large flight arena to observe the movement of bees and operate the Bee-tracker software (developed in collaboration with James Elderfield and Dr Nik Cunniffe, University of Cambridge, Cambridge, UK) for records. Each experiment lasted for three hours at most. I did not have control over the number of visits allowed per plant. Hence the decision to limit the experimental time to not more than three hours.

'Bee-tracker' software was developed to record the movements and of bumblebees and time spent on individual flowers at plant level, to allow subsequent visualization and analysis of data. When a bee lands on a flower, a "Start experiment" button could be clicked to allow the timer to

begin recording. The grey button under appropriate plant number was used to record the duration that the bees were pollinating on each bean plant. After a bee had made it's first ten choices, the program could be closed, and it would automatically save all the recorded data. The output files were statistically analysed in R.

All trip-pollinated flowers were marked with non-toxic paint on the flower stalk. The plants were allowed to grow further in the glasshouse, and mature pods were harvested, counted and recorded. Seeds from trip-pollinated and self-pollinated flowers were harvested from their pods, counted and recorded. Each experiment was repeated three times for each virus, BCMV and CMV.

2.9 Outdoor pollination experiments

Initial trials of this experiment were done in the summers of 2016 and 2017, which led to the modification of methods described herein. Mock-inoculated and virus-infected plants were raised in the glasshouse and transplanted *insitu* (Cambridge University Botanic Garden experimental plots) five days post-inoculation. They were immediately covered with an aphid-proof mesh (0.3 mm X 0.7 mm mesh size, Wondermesh Limited, Scotland) (Figure 2.8A). Upon flowering, half the batch of plants was opened-up for pollinators to forage on them (bee-pollinated treatment) and the other half batch remained completely covered to allow self-pollination (self-pollination treatment). The opened-up netting only covered the top, with sides left open (Figure 2.8B). This was done to control the effect of shading on plant growth so that both treatments (insect-pollinated and self-pollinated plants) would have netting shade factor on them. A batch of back-up plants (in case the garden plants were destroyed by biotic and abiotic factors) was raised in the glasshouse in 4-litre black pots. These were moved to the Botanic Garden experimental plot upon flowering. The plants were covered as described above. Observations of pollinators were done in the same way as for plants growing in the experimental plot, as described on Page 64.

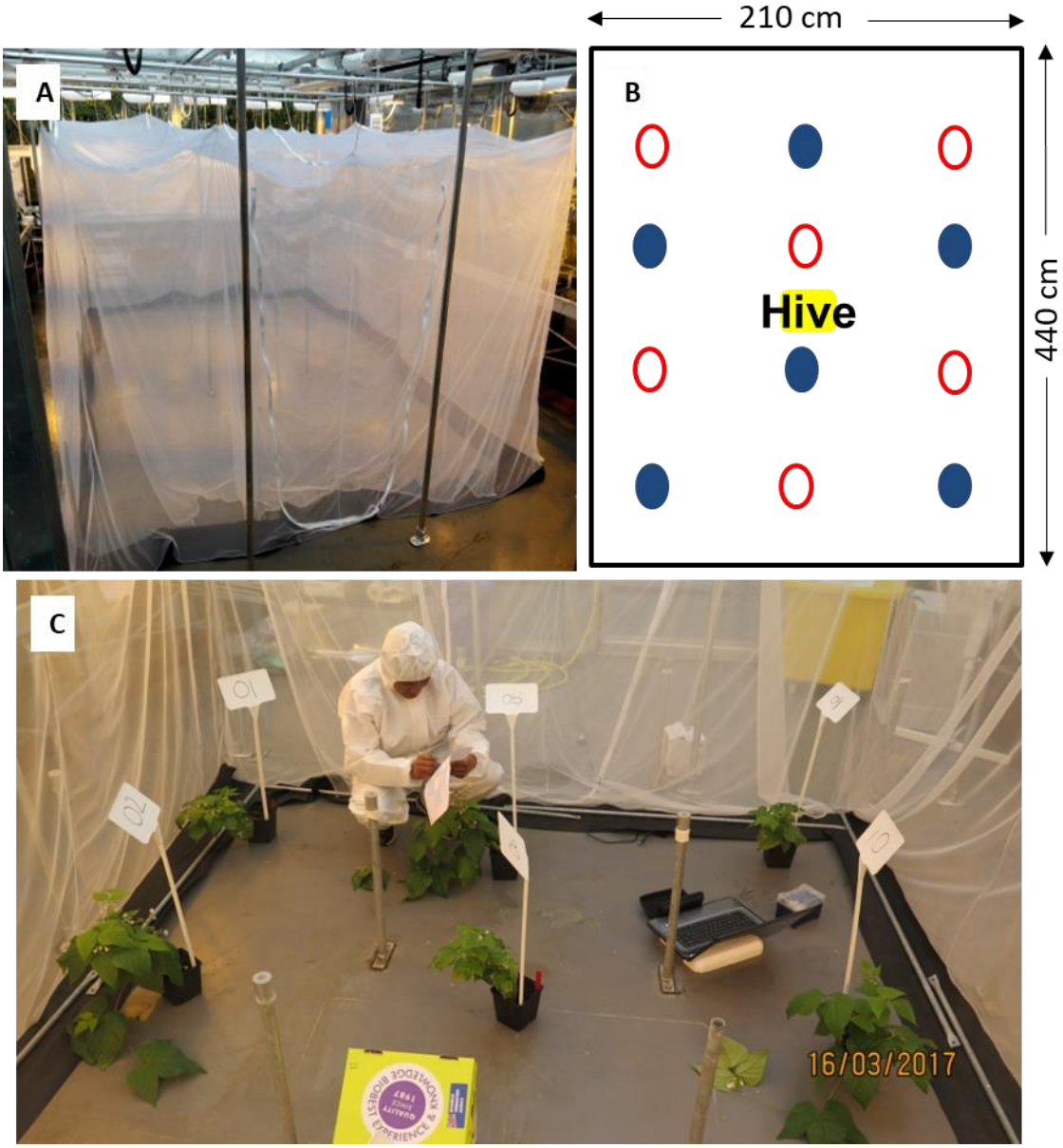


Figure 2.7 Flight arena used for pollination experiments in the glasshouse. (A) A large flight arena (320 x 440 x 210 cm, H x W x D) was constructed out of nylon netting with a front zipped door to allow full access of plants and people. (B) Cartoon demonstrating the arrangement of mock-inoculated (blue dot) and CMV-infected plants (red circle) within the larger flight arena. (C) A bumblebee colony was introduced into the large flight arena with 6 mock-inoculated and 6 virus-infected flowering common bean plants. The author is shown collecting data.


A

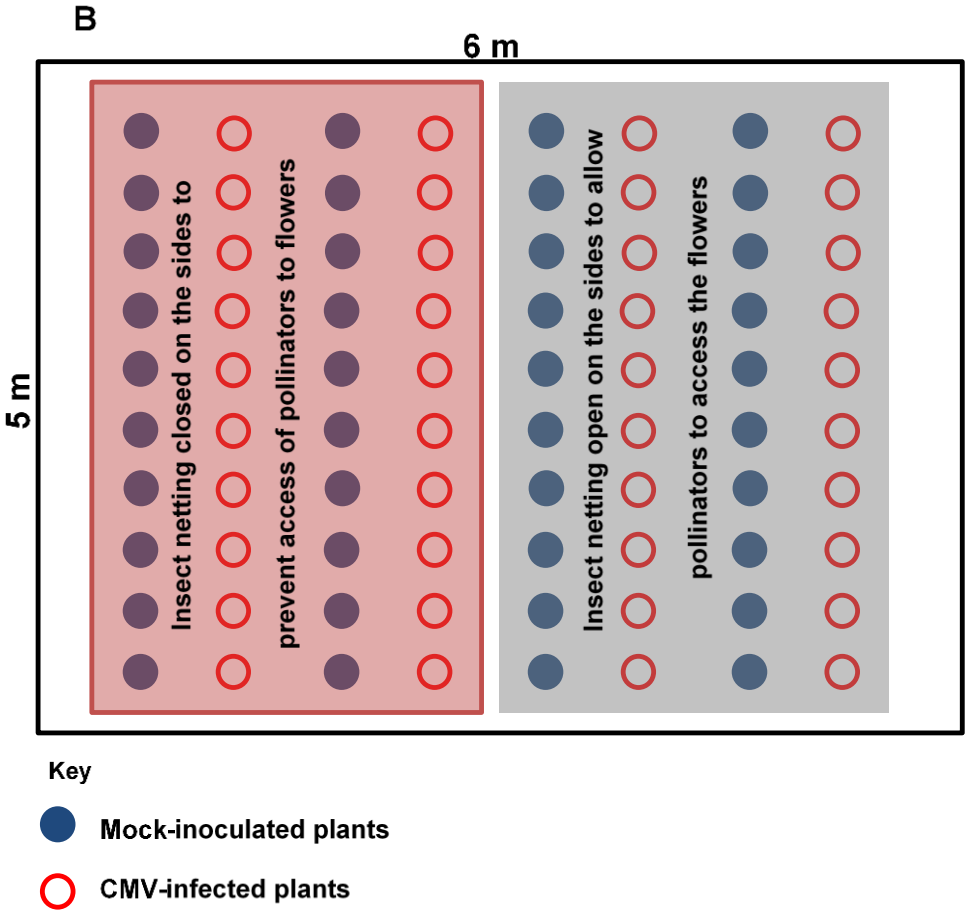

B
6 m
5 m
Insect netting closed on the sides to prevent access of pollinators to flowers
Insect netting open on the sides to allow pollinators to access the flowers
Key
Mock-inoculated plants
CMV-infected plants

Figure 2.8 Experimental set-up in the Cambridge University Botanic Garden experimental plot measuring 6 m X 5 m. (A) Plants were grown under an aphid-proof mesh to prevent the potential entry of virus-bearing aphids. (B) Upon flowering, half the batch of plants was opened up on the side to allow pollinators to forage on flowers and the other half remained closed off on all sides to exclude pollinators as shown in the schematic diagram. (C) Pollinators were observed and identified and their foraging behaviour appearance, and their pollination behaviour noted. Frequency of visits of legitimate pollinators was recorded between 10:00 am and 2:00 pm for 10 days. The author is shown collecting data.

Initial extensive observations of the activities of floral visitors were undertaken during daylight hours (05:00–20:00 hours) for two days to identify any specific peak periods when pollinators would visit the flowers the most. However, there were no such peak periods and pollinators were observed at all times between 06:00 and 18:00 hrs. Therefore, 10:00 – 14:00 hrs was chosen as the standard observation period (Figure 2.8B) for a period of 10 days. All pollinators were identified by their appearance using a field guide by Falk (2015), and their pollination behaviour was noted. Nature of nectar collection was regarded as either legitimate (via the corolla mouth) or robbing (chewing a hole through the corolla) (Inouye 1980; reviewed in Irwin *et al*. 2010). The frequency of visitation of legitimate pollinators both to virus-infected and mock-inoculated plants was recorded. At the end of the flowering period, the insect mesh was removed. Plants were allowed to continue growing until fruits matured, where after the mature pods were harvested, counted and their seeds counted per plants per treatment. Fruit set was recorded as the number of pods per plant and yield measured as the total number of seeds per plant.

2.10 General Statistical Analyses

All the sample sizes used in data collection were chosen based on statistical power. Thus, the number of replicates used was above the minimum number that could give satisfactory statistical power. Statistical analyses were carried out in R (R Core Team, 2015). Mean and standard error of the mean (SEM) were computed for bar graphs, and the graphs were generated in Microsoft® Excel. Error bars in all graphs represent the SEM. Nectar datasets were checked for normality using R. Datasets that did not follow a normal distribution were transformed using R. Normalised data were analysed using one-way analysis of variance (ANOVA), and subsequently compared using Tukey's honest significance test (HSD) test. Bumblebee free assay choice tests were analysed by a binomial test to determine whether bumblebee preference significantly differed from an expected frequency distribution of 50:50 for either mock-inoculated or virus-infected bean plants. Seed yield data from glasshouse and Botanic Garden pollination experiments were

analysed using two-sample t-tests. All p values < 0.05 were considered to be statistically significant.

CHAPTER 3

VIRUSES INDUCE CHANGES IN FLORAL TRAITS OF COMMON BEAN

3.1 INTRODUCTION

Bee pollinators are guided to suitable flowers by multimodal sensory signals summarised in Section 1.3. Bumblebees can effectively learn to associate particular floral features with nectar and pollen rewards (Cnaani *et al*. 2006; Raine *et al*. 2006; Gomez *et al*. 2008; Konzmann & Lunau 2014). Hence floral architecture is important in plant-pollinator interactions. In this chapter, I investigated the effects of BCMV, BCMNV and CMV on common bean floral traits that are considered to be important determinants of pollinator preferences. The floral observations and measurements (Table 2.1 and Section 2.5 – 2.6) provide baseline information on the likely behaviour of bees given the choice of visiting healthy or virus-infected plants.

3.2 RESULTS

3.2.1 BCMV, BCMNV and CMV induced stunting and mosaic symptoms in common bean

The initial step of my experiments was to inoculate common bean cv. Wairimu plants and *V. faba* plants with BCMV, BCMNV and CMV. Mechanical sap inoculation was used to infect the plants and control plants were mock-inoculated with sterile water (Section 2.3). The plants were observed for two weeks, and symptoms that developed were documented by photography, and the presence of viruses in the infected plants was confirmed by RT-PCR (see Section 2.4). All three viruses induced stunting, leaf deformity and mosaic patterns in common bean (Figure 3.1). The presence of viral RNA in infected plants was confirmed by RT-PCR (Figure 3.3). The opposite was observed in *V. faba* plants, whereby all the three viruses did not induce any symptoms in

inoculated plants (Figure 3.2). The inoculations were repeated three more times, but the *V. faba* plants remained symptom-free, and the three viruses could not be detected in inoculated plants by RT-PCR (Figure 3.3). This suggested that *V. faba* varieties Fuego and Tatoo are not hosts for the BCMV, BCMNV and CMV strains in use in our laboratory.

3.2.2 BCMV, BCMNV and CMV reduce flower numbers and BCMNV delays flowering in common bean

Mock-inoculated and virus-infected common bean cv. Wairimu plants were grown and monitored until the first floral buds emerged. Records of the number of days the plants took to develop first floral buds from seed imbibition date and inoculation date were kept. CMV-infected plants produced their first floral buds around the same dates as mock-inoculated plants. The onset of flowering was significantly delayed by about 2-5 days in BCMV-infected plants and 8-11 days in BCMNV-infected plants (Table 3.1).

Counts of flowers and buds present on the seventh day from the onset of flowering were done across all treatments. Forty plants from each treatment were used for flower counts. All three viruses (BCMV, BCMNV and CMV) caused a significant decrease in the number of flowers (Figure 3.4). On average, a mock-inoculated plant produced an average of 22 flowers by the seventh day into flowering, a BCMNV or CMV-infected plant produced an average of 12 flowers and a BCMV-infected plant an average of 14 flowers (Figure 3.4).

3.2.3 Effects of BCMV, BCMNV and CMV on flower morphology

Newly opened flowers (Figure 2.3A) were photographed and measurements taken (see Section 2.5.1 & Figure 2.3B) to assess the traits that a bee visiting a flower would encounter in the field. Measurements were made within the second week of flowering across all treatments. The aim was to determine if BCMV, BCMNV or CMV infection cause changes in flower dimensions (length

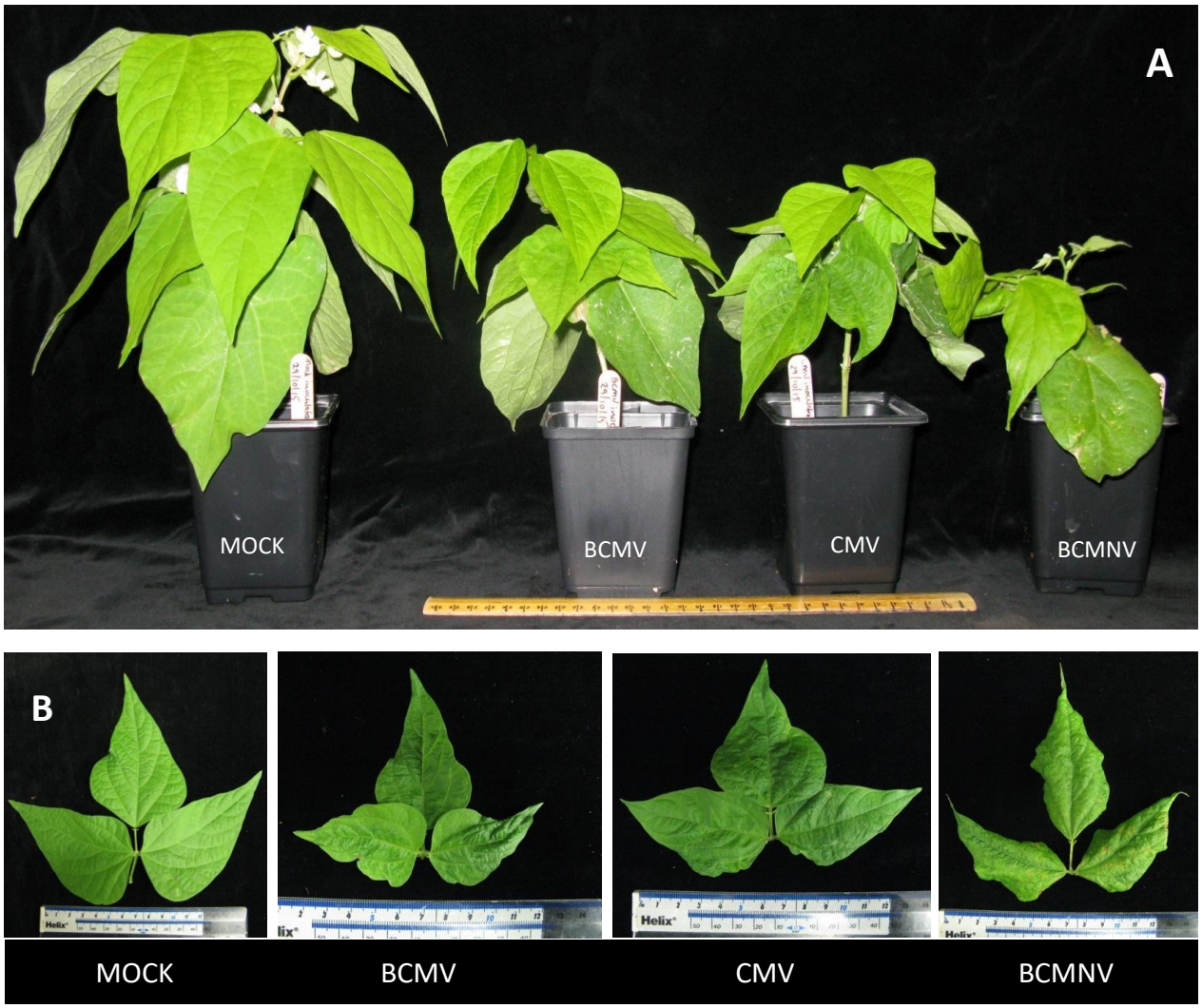


Figure 3.1 Symptoms caused by BCMV, BCMNV and CMV on *P. vulgaris* cv. Wairimu. (A) All the virus-infected plants were stunted in growth when compared to the mock-inoculated plant. BCMNV-infected plants had the most severe stunting. (B) All three viruses caused visible symptoms to the upper trifoliate leaves. Symptoms were characterised by leaf curling, rugosity, mosaic pattern and vein clearance. BCMNV-infected leaves had yellow to brown necrosis spots as well. Trifoliate leaf from mock-inoculated plants shows no signs of deformation. All photos were taken at 22-26 dpi. Scale bars (A) = 30 cm.

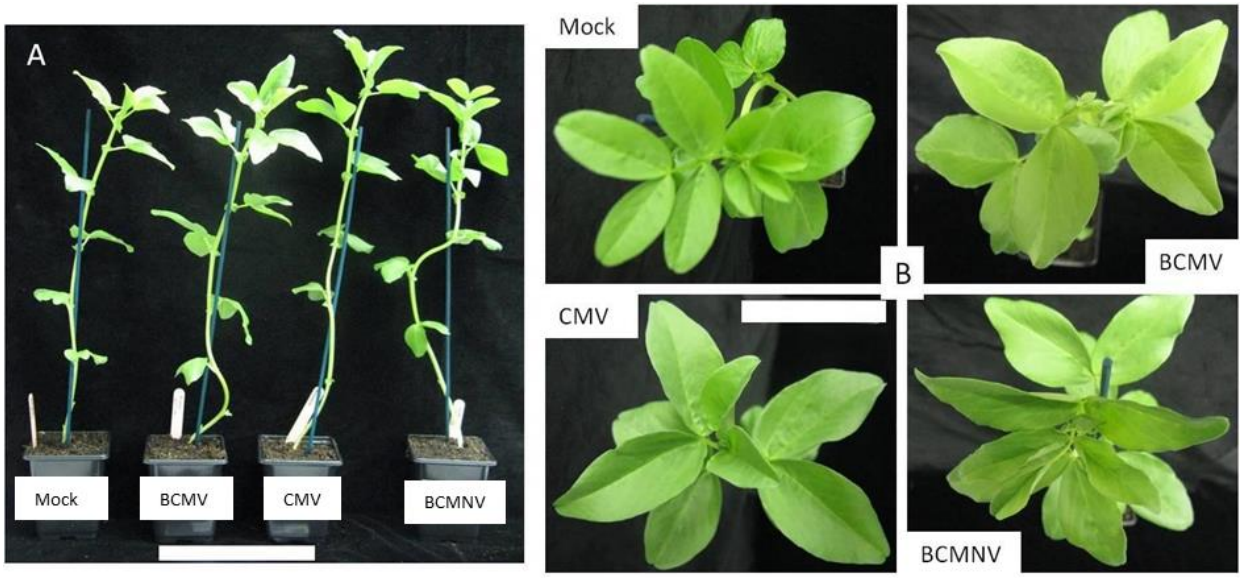


Figure 3.2 *Vicia faba* is not susceptible to our lab strains of BCMV, BCMNV or CMV. Plants were inoculated with the viruses indicated. *V. faba* cv. Tatoo (A) and *V. faba* cv. Fuego (B) plants showed no apparent symptoms of virus infection. Photographs were taken at 14 days post-inoculation. Scale bars (A) = 15 cm and (B) = 4.5 cm**.**

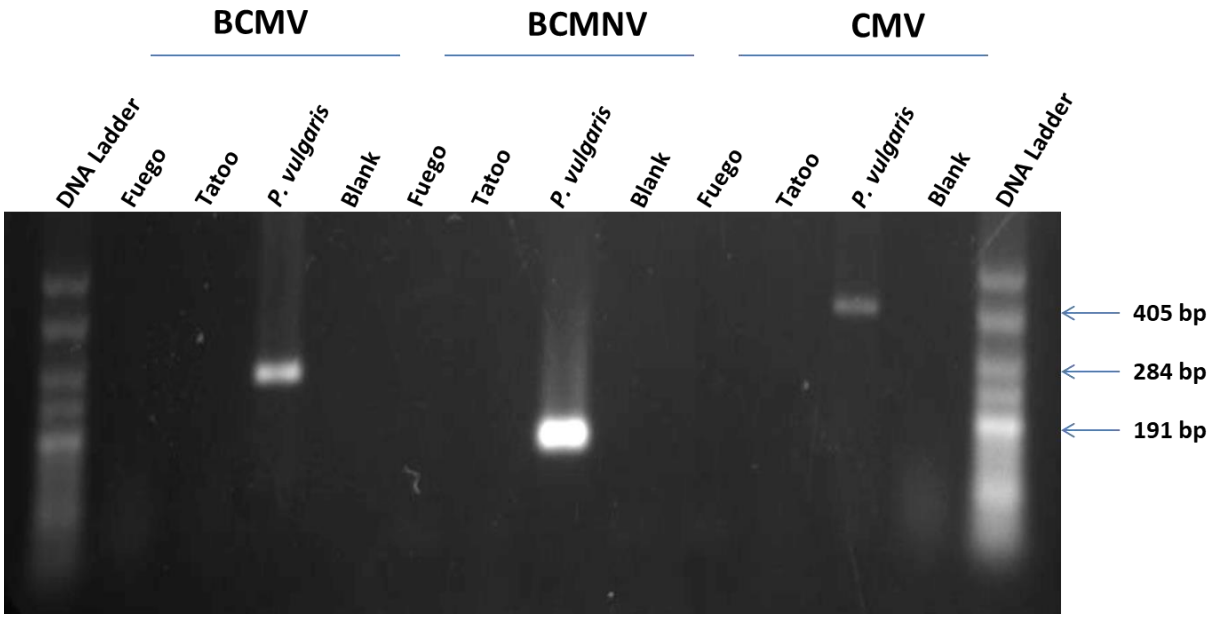


Figure 3.3 Viral RNA detection using RT-PCR of RNA from leaves of virus-inoculated *Vicia faba* and *Phaseolus vulgaris* cv. Wairimu plants. A 1.5% agarose gel was run to check if the PCR products amplified using coat protein gene-specific primers of BCMV, BCMNV and CMV were present. The size of amplified cDNA fragments was estimated with 500 base pairs (bp) ladders. Blanks were loaded with reactions in which sterile water replaced cDNA. 'Fuego' and 'Tatoo' are cultivars of *V. faba*. The *P. vulgaris* cultivar used here was 'Wairimu.' Blank reactions are negative controls that do not have cDNA and are used to check if there is any contamination of undesired DNA in the samples.

Table 3.1 Number of days cv. Wairimu plants took to produce the first floral buds across all treatments.

Treatment	Start of flowering days post inoculation (dpi)
Mock-Inoculated	18-20
Bean common mosaic virus (BCMV)	20-23* (p = 0.009)
Bean common mosaic necrosis virus (BCMNV)	29-32* (p = 0.0001)
Cucumber mosaic virus (CMV)	19-23

One-way ANOVA was used to test for significant differences in the number of days it took for plants to produce their first floral buds from inoculation date [$F(3,156) = 725.5$, $p = 1.1102 \times 10^{-16}$; $n = 160$]. The p-values given are from Tukey's HSD post-hoc tests comparing each virus treatment with mock-inoculated plants.

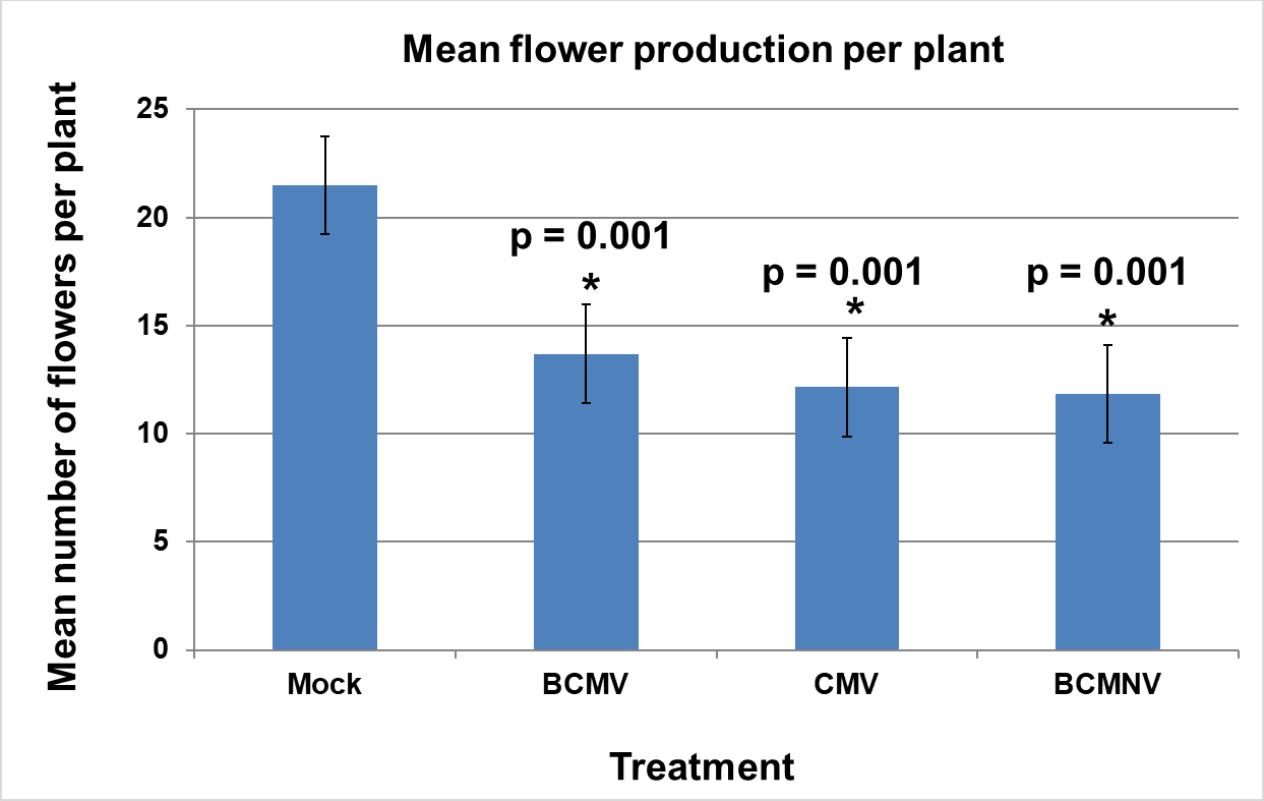


Figure 3.4 Virus infection decreases the number of flowers produced by cv. Wairimu plants. Flower numbers per plant were counted within the second week from the onset of flowering. A one-way ANOVA was done on the flower number dataset and results showed that BCMV, BCMNV and CMV-infected common bean plants produced significantly fewer flowers in comparison with mock-inoculated plants [$F(3,156) = 68.45$, $p = 1.1 \times 10^{-16}$; $n = 160$]. Error bars represent the standard error of the mean. * Represents significant statistical difference to mock treatment using Tukey's HDS multiple comparisons of means at 95% family-wise confidence level.

or petal area). Generally, flowers from virus-infected plants were slightly smaller than flowers from mock-inoculated plants, the smallest being from CMV-infected plants (Figures 3.5 & 3.6). Flowers from CMV-infected plants were the smallest in length (mean = 23.85 mm) (Figure 3.6A). Flowers from BCMNV-infected plants had an average length of 24.38 mm, and those from BCMV-infected plants were 24.92 mm long (Figure 3.6A).

Flowers produced by mock-inoculated plants were slightly longer than those from virus-infected plants (Figure 3.6A). There were no significant statistical differences in the length of flowers from all virus-infected plants in comparison to flowers from mock-inoculated plants. Flowers from all virus-infected plants had corolla tube mean lengths of 8.0 mm whereas mock-inoculated plants produced flowers with corolla tube mean lengths of 8.5 mm. This difference was not statistically significant (Figure 3.6B).

There were no significant differences in petal standards among all treatments, although their sizes varied. Standard petals from BCMV-infected plants had the smallest size mean area of 104.43 mm^2, whereas standard petals from BCMNV and CMV-infected plants (mean areas = 106.02 mm^2 and 105.84 mm^2 respectively) were almost the same size as those from mock-inoculated plants (mean area = 106.91 mm^2) (Figures 3.5 and 3.6C).

BCMV-infected plants produced flowers with the biggest wing petals (mean area = 91.57 mm^2), followed by those from mock-inoculated plants (mean area = 89.12 mm^2) (Figure 3.6D). BCMNV and CMV-infected plants produced the smallest wing petals of them all (mean areas = 85.68 mm^2 and 85.15 mm^2, respectively) (Figure 3.6D). However, wing petal sizes were not statistically different across all treatments (Figure 3.6D).

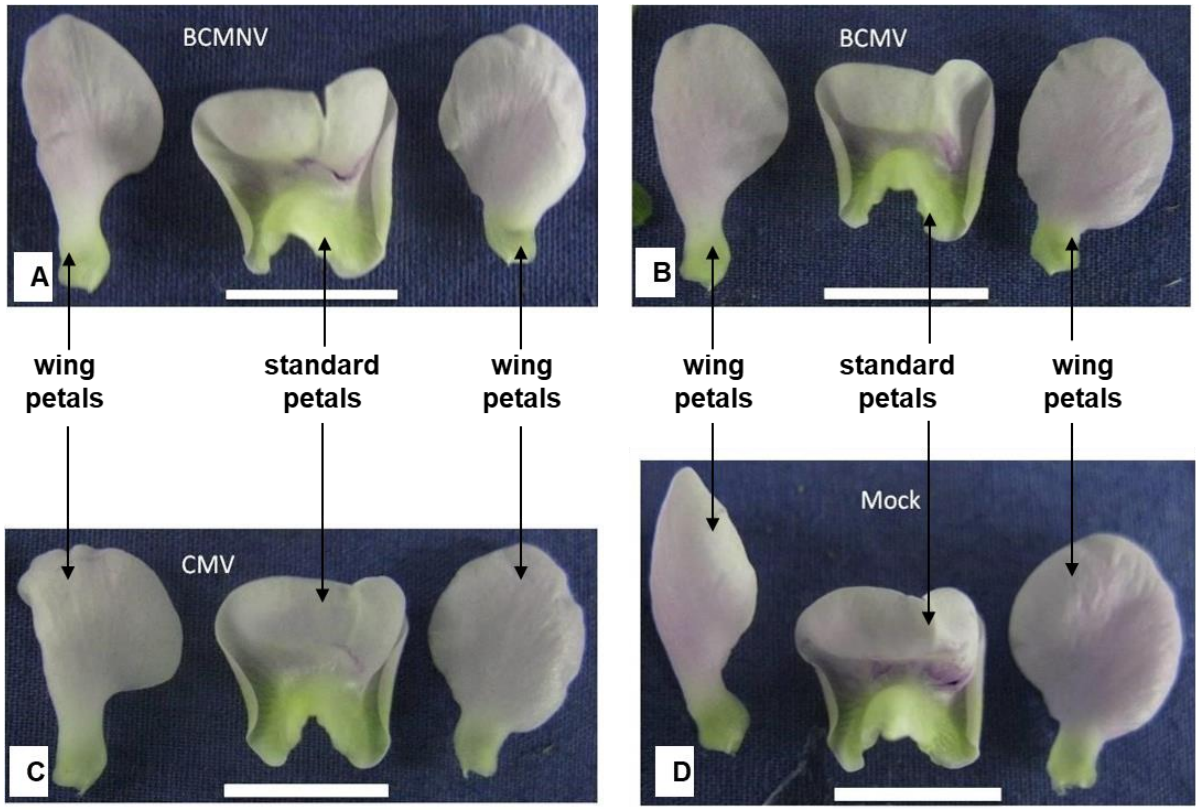


Figure 3.5 Effects of virus infection on *P. vulgaris* cv. Wairimu flower petal size. Petal size measurements were calculated as described in Section 2.5.1 and illustrated in Figure 2.3B. Standard and wing petals from mock-inoculated flowers (D) were slightly bigger than those from (A) BCMNV-infected plants; (B) BCMV-infected plants; and (C) CMV-infected plants. Standard of BCMV-infected plants (B) was markedly reduced in size. Statistical analysis results are given in Figure 3.6. Scale bars = 20 mm.

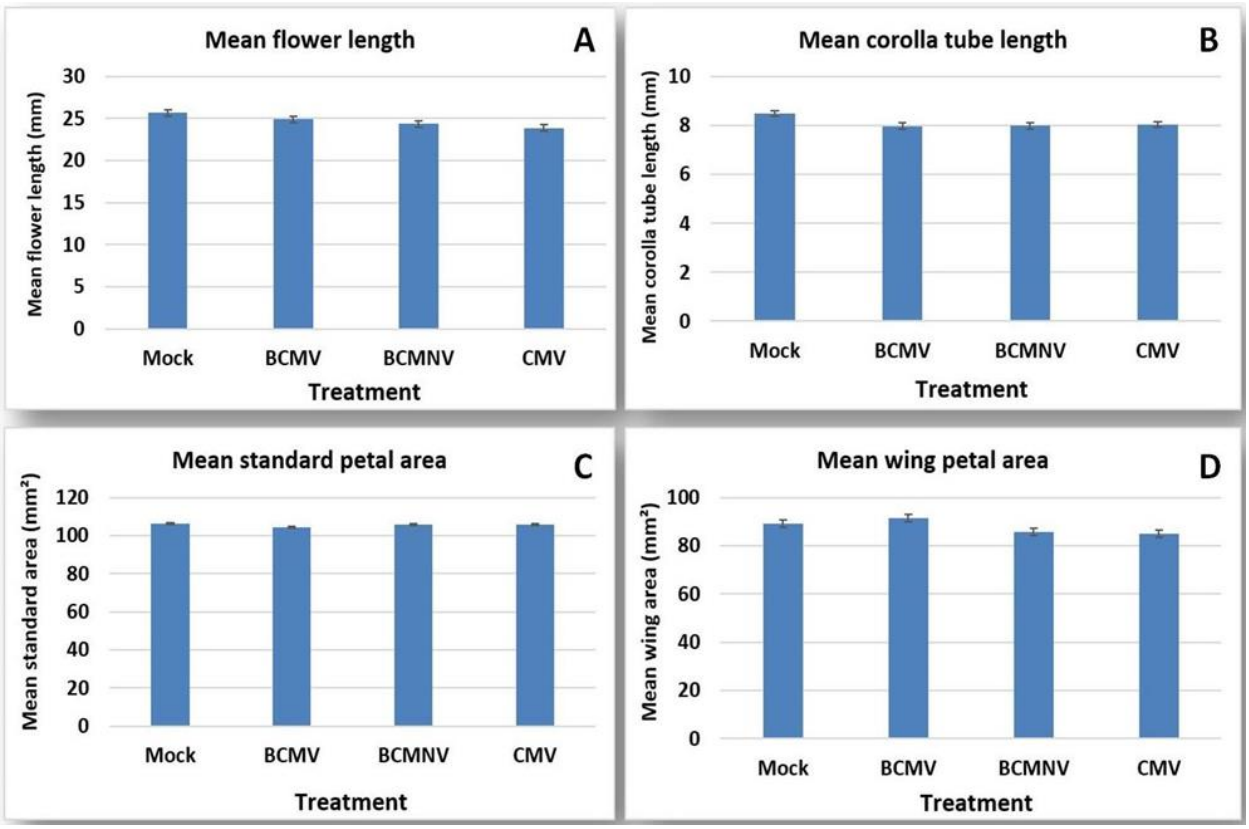


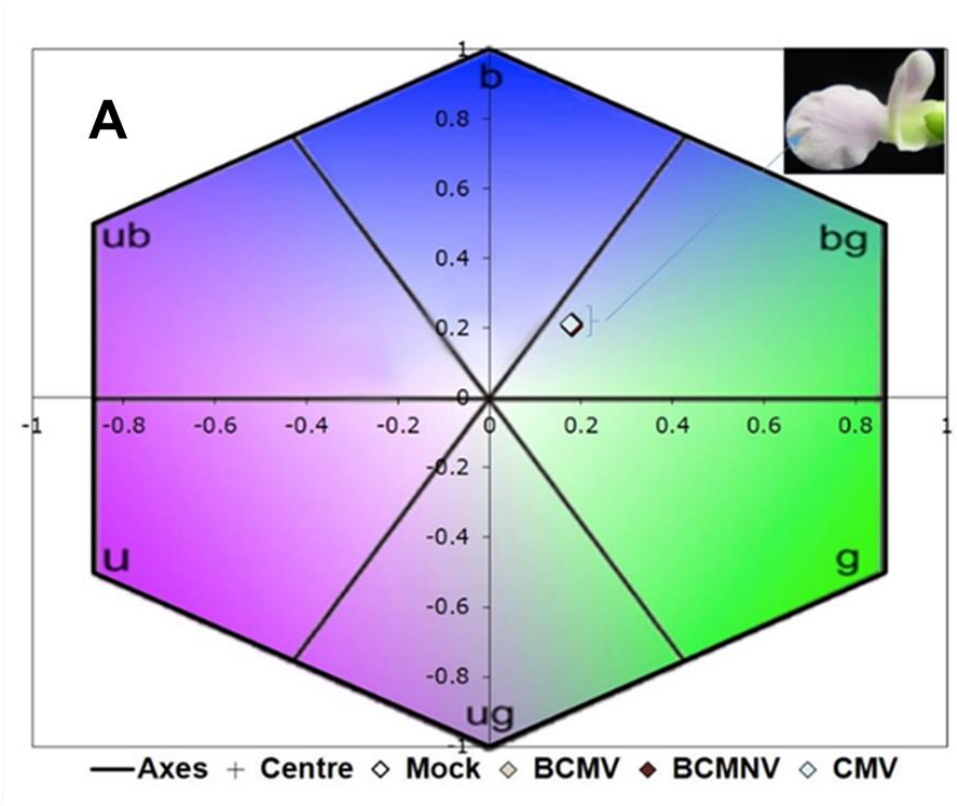
Figure 3.6 Effect of virus infections on flower dimensions in cv. Wairimu plants (pictures shown in Figure 3.5). Floral morphology results represented here in these graphs were measured and calculated as described in Section 2.5.1 and illustrated in Figure 2.3B. (A) Flowers from virus-infected plants appeared to be slightly shorter in length than flowers from mock-inoculated common bean plants. There were no significant differences in the flower lengths as determined by one-way ANOVA [F (3, 116) = 2.37, p = 0.075, n = 120]. (B) Virus-infected plants produced flowers with slightly shorter appearing corolla tube lengths when compared to flowers from mock-inoculated plants in common bean plants. However, there were no significant differences in the corolla tube lengths of the flowers across all treatments [one-way ANOVA: F (3, 116) = 2.1, p = 0.11, n = 120]. (C) Areas of standard petals across all treatments were not statistically different [one-way ANOVA: F (3, 116) = 0.086, p = 0.97, n = 120]. Standard petals of flowers from BCMV-infected plants were the smallest. (D) Flowers from BCMV-infected plants had slightly bigger wing petals among all treatments. However, there were no significant differences

in the wing petal area (one-way ANOVA: [F (3, 116) = 2.09, p = 0.11, n = 120]. Error bars represent the standard error of the mean.

3.2.4 Effects of virus infection on floral colour of cv. Wairimu as perceived by bees

The colour of cv. Wairimu flowers was measured using a spectrometer at two points on the pollinator-facing surfaces across all treatments (Figure 3.7): the abaxial side of the wing tip and the adaxial side of the standard petal on the nectar guides. The reflectance spectra were then converted into coordinates in the colour hexagon of bee visual space (Chittka, 1992). To human eyes, the wing petal appears light purple and the adaxial side of the standard petal appears purple, which becomes slightly darker at the nectar guides (Figure 3.7). Both flowers petals appear blue-green to a bee (Figure 3.7), but each petal occupies different positions in the blue-green region in the hexagon. All three viruses caused a slight colour change on the nectar guides on the adaxial face of standard petals of flowers of cv. Wairimu plants as indicated by hexagon units and positions in the hexagon (Figure 3.7B and Table 3.2). The opposite is true for wing petals of cv. Wairimu whereby none of the viruses induced a colour change (Figure 3.7A). All treatments clustered together occupying the same position in the bee visual space hexagon (Figure 3.7B).

The average distances between positions occupied by samples from mock-inoculated and CMV-infected plants, mock-inoculated and BCMNV-infected plants, and mock-inoculated and BCMV-infected plants along the x-axis are 0.049, 0.033 and 0.023 hexagon units, respectively, where one hexagon unit represents the distance between the centre of the hexagon (0 unit which means no excitation) and any point on the boundaries of the hexagon (1 unit which means maximal excitation) (Table 3.2). Honey bees can discriminate between colours as little as 0.008 hexagon units apart with 75% accuracy (Dyer & Neumeyer 2005). Thus, the colour changes induced by all the three viruses on nectar guides on standard petals are perceivable by honeybees. However, bumblebees have poor abilities than that of honey bees, discriminating the same colour pair as above with less than 60% accuracy (Dyer 2006). It will be unlikely that bumblebees would perceive these colour differences.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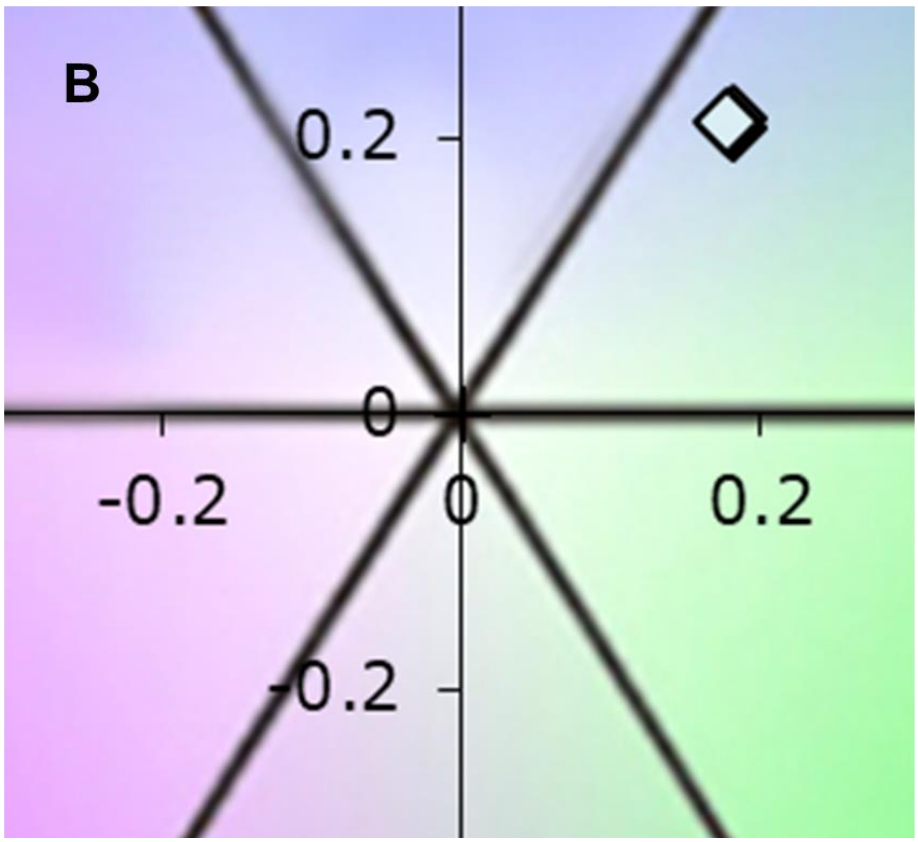
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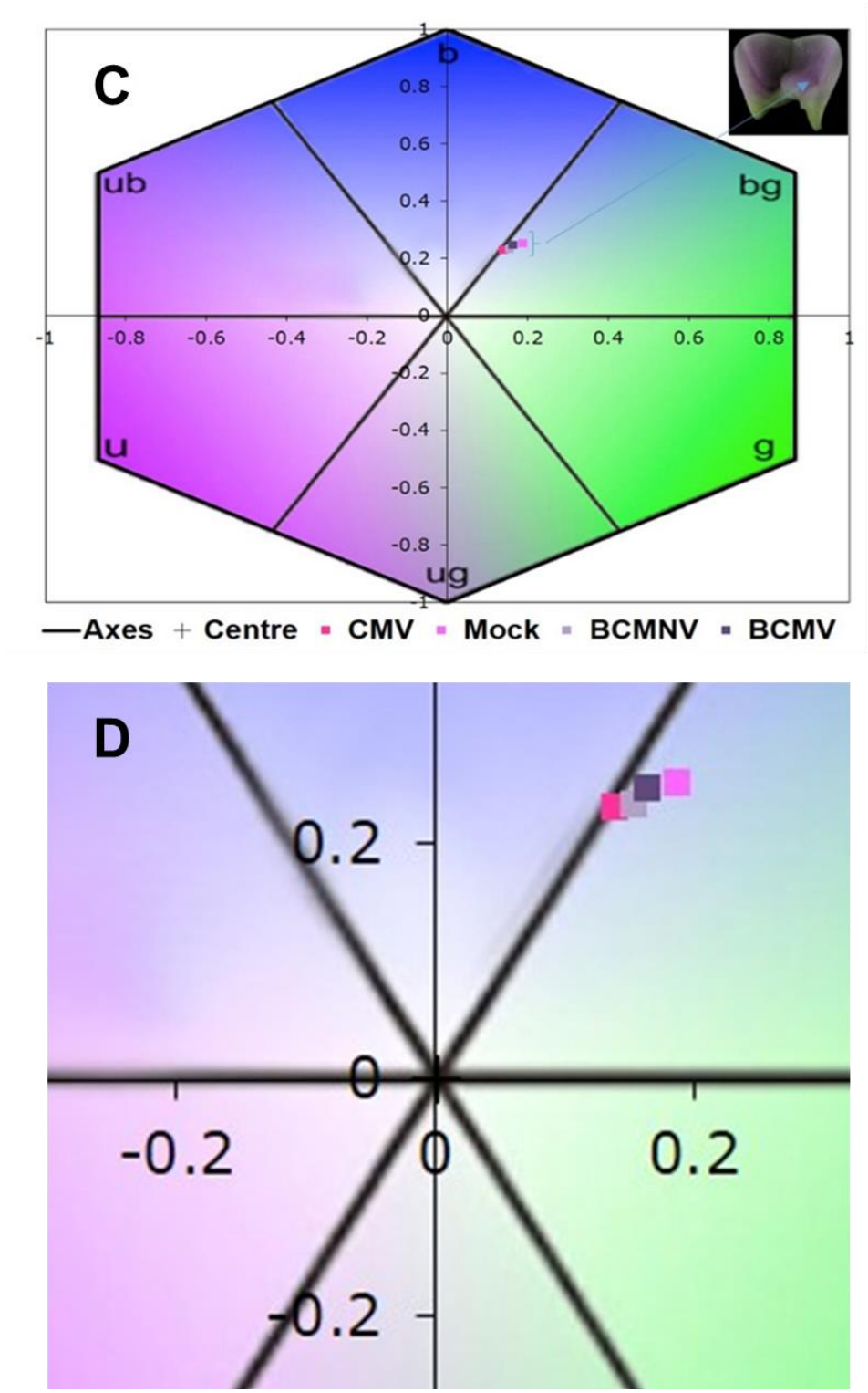


Figure 3.7 Influence of virus infection on the distribution of *P. vulgaris* cv. Wairimu floral petal colour in bee space. (A) Wing petal tip colour as perceived by bees. All treatments are clustered at the same position in the blue-green region. (B) A zoomed-in picture showing clustered

positions occupied by all treatments in the bee space hexagon. (C) Standard petal nectar guide colour as perceived by bees. Each treatment occupies a different position in the blue-green region, but with little difference from the control. Samples from CMV-infected plants occupy a position furthest from mock-inoculated plants. The centre of the hexagon represents achromatic colours such as bee-white or bee-black, which means no excitation responses from the trichromatic photoreceptors of the bees. (D) A zoomed-in picture of (C). Scale: 1 hexagon unit is the distance from the centre of the hexagon to any of its boundaries.

Table 3.2 Hexagon units (x and y-axis) for standard petals for all treatments

Treatment	X-axis (Mean, SE)	Y-axis (Mean, SE)
CMV	0.138, 0.011	0.231, 0.012
Mock	0.187, 0.019	0.253, 0.012
BCMNV	0.154, 0.012	0.234, 0.0071
BCMV	0.164, 0.009	0.247, 0.0056

Table 3.3 Hexagon units (x and y axis) for wing petals for all treatments

Treatment	X-axis (Mean, SE)	Y-axis (Mean, SE)
CMV	0.192, 0.005	0.202, 0.0039
Mock	0.187, 0.0065	0.198, 0.0031
BCMNV	0.184, 0.0063	0.203, 0.0034
BCMV	0.185, 0.0059	0.205, 0.0051

3.2.5 Effects of virus infection on epidermal cell morphology of cv. Wairimu flowers

The epidermal cell morphology of flowers was assessed for the abaxial surfaces of wing petals using SEM. Ojeda *et al*. (2009) categorized six main cell types present in Fabaceae (Figure 1.3) based on three levels: the shape of the cell perimeter (perimeter shape); the amount of projection from the cell surface (projection); and the micromorphology of the cell surface (Cell surface micromorphology). Only the wing petals were assessed because these are the petals that bees land on and grip onto when collecting nectar from bean flowers. Of particular interest to this project were the papillose conical cells (Section 1.3 & Figure 1.3) (herein termed conical cells) that have been shown to influence pollinator preference (Whitney *et al*. 2009; Alcorn *et al*. 2012). The cell surface micromorphology of these cells can either be smooth, striate or granular. The purpose was to find out if virus infections induce changes in these cells. I observed conical cells with striated cell surfaces across all treatments. There were no notable changes on the morphology of these conical cells on the abaxial side of wing petals of flowers of mock-inoculated and virus-infected plants (Figure 3.8).

3.2.6 Under controlled conditions viruses cause changes in nectar produced by cv. Wairimu

Nectar was extracted by centrifugation from fully open flowers (section 2.5.4) between 10:00 and 12:00 hours from plants in their second week of flowering across all treatments. These data were obtained from 400 flowers (80 plants), that is, 100 flowers from 20 of each of the treatments. Volume of nectar was calculated using the equation in Section 2.5.4 of Chapter 2. Nectar was collected from plants grown in controlled conditions and glasshouse.

BCMV-infection of cv. Wairimu plants induced a significant increase in floral nectar production, whereas CMV and BCMNV-infection did not induce any nectar production changes (mean values per flower: Mock = 2.2 µl; BCMV = 2.6 µl; BCMNV = 2.2 µl; and CMV = 2.5 µl) in plants grown in

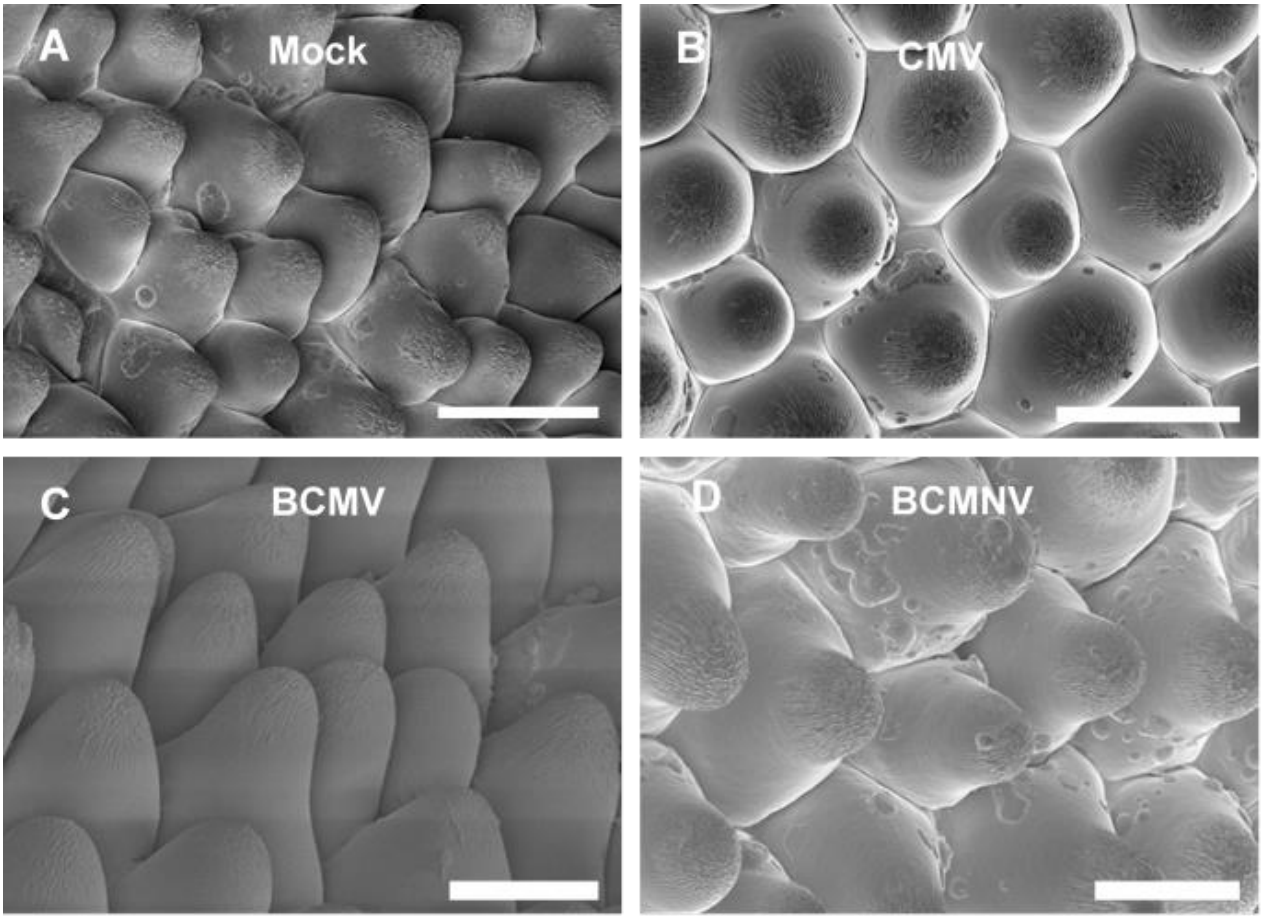


Figure 3.8 Morphology of conical epidermal cells on the abaxial face of wing petals in cv. Wairimu. The cells have the same morphology (papillose conical striate) across all treatments which were (A) from mock-inoculated plants; (B) from CMV-infected plants; (C) from BCMV-infected plants; and (D) from BCMNV-infected plants. Scale bars (A) = 40 µm, (B) = 30 µm, (C) and (D) = 20 µm.

controlled conditions (Figure 3.9A). Further post-hoc Tukey HSD comparisons were made, each virus treatment versus mock-inoculated treatment. Flowers from BCMV-infected plants proved to have nectar volume that was significantly higher than those from mock-inoculated plants (Figure 3.9A). Nectar sucrose concentration was increased by presence of viruses in cv. Wairimu plants bean (Means: Mock = 23%; BCMV = 26%; BCMNV = 25% and CMV = 27%) (Figure 3.9B). Significant differences in nectar sucrose concentration were observed in flowers from BCMV and CMV-infected plants as confirmed by One-way ANOVA post-hoc Tukey's HSD comparisons with flowers from mock-inoculated plants. For plants grown in the glasshouse, BCMV and CMV infection did not induce any change in flower nectar production (Means: Mock = 3.7 µl; BCMV = 3.6 µl and CMV = 3.4 µl) (Figure 3.10A). BCMV infection did not induce any change in sucrose concentration, whereas CMV infection caused a significant decrease in floral sucrose nectar concentration (Means: Mock = 25%; BCMV = 26%; and CMV = 23%) (Figure 3.9B). Significant differences were confirmed by post-hoc comparisons with flowers from mock-inoculated plants. BCMNV was not used in glasshouse experiments because it is a DEFRA licenced pathogen with strictly restricted usage in contained laboratory enclosures.

3.2.7 Viruses alter volatiles emitted by common bean flowers

Results from the GC-MS analysis (Section 2.6) revealed qualitative and quantitative changes in floral volatile blends in response to virus infection. Principal component analysis of the mass spectra was performed using MetaboAnalyst 4.0 (Xia *et al*. 2012). Results revealed overall qualitative changes in the volatile blends of virus-infected plants (Figure 3.11). The VOCs blend from mock-inoculated plants clustered separately from those of virus-infected plants (Figure 3.11).

The quantity of VOCs emitted by flowers from BCMNV and CMV-infected plants were increased in five most abundant compounds, namely phenol, linalool, palmitic acid tetradecane and an unknown compound (Figure 3.12A). Further analysis showed linalool emission was increased in

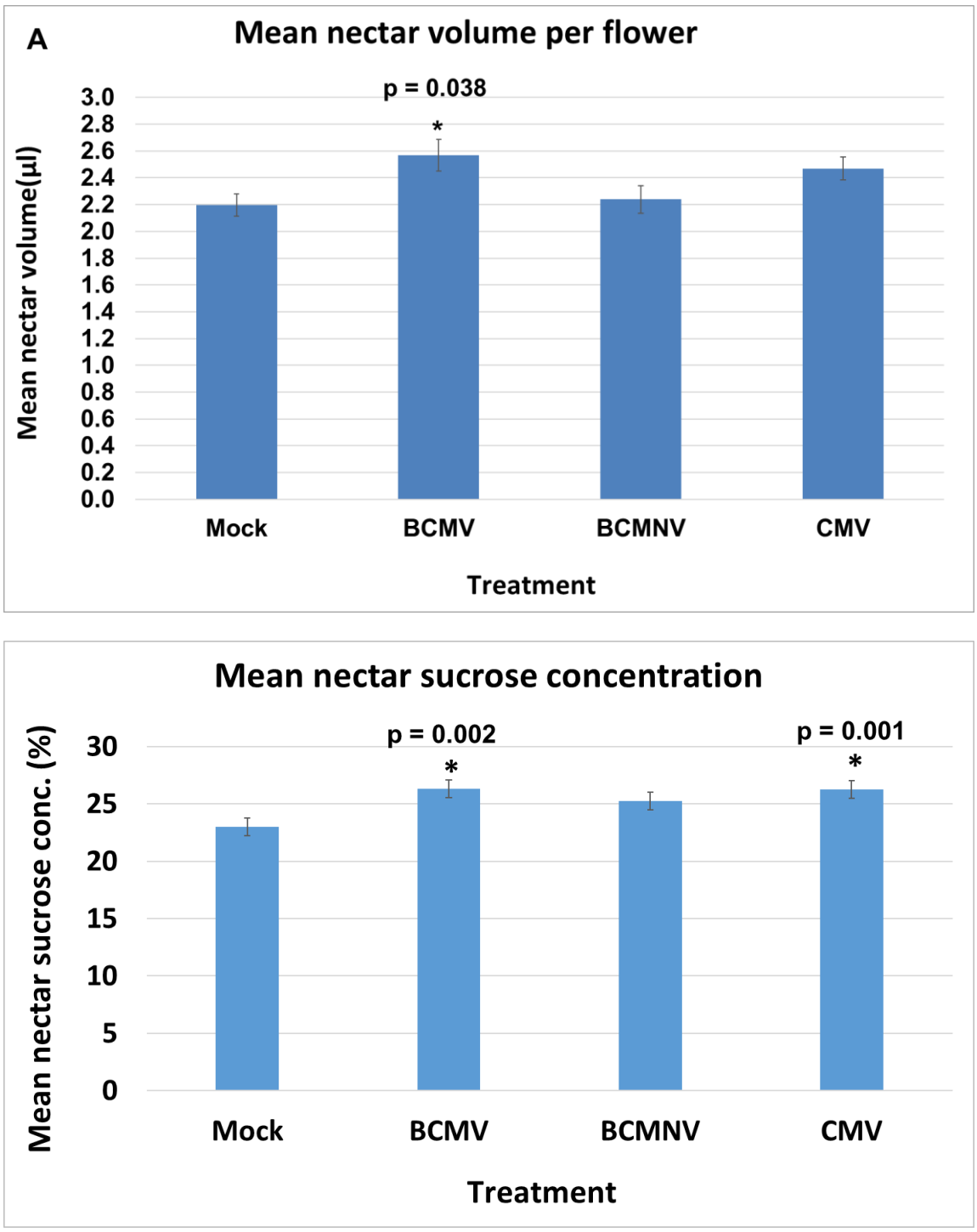


Figure 3.9 Effects of viruses on floral nectar production in cv. Wairimu under controlled PGF conditions. (A) BCMV-infected plants produced flowers with mean nectar volume per flower significantly different from that of mock-inoculated plants [one-way ANOVA: F (3, 396) = 3.3585, p = 0.019]. (B) Flowers from plants infected with BCMV and CMV produced nectar with

significantly higher sucrose concentration in comparison to that from flowers of mock-inoculated plants [One-way ANOVA: F (3, 396) = 7.873; p = 5.12 x 10-5]. Error bars represent the standard error of the mean. * Represents significant statistical difference to mock treatment using Tukey's HDS multiple comparisons of means test at 95% family-wise confidence level as indicated by the p-values.

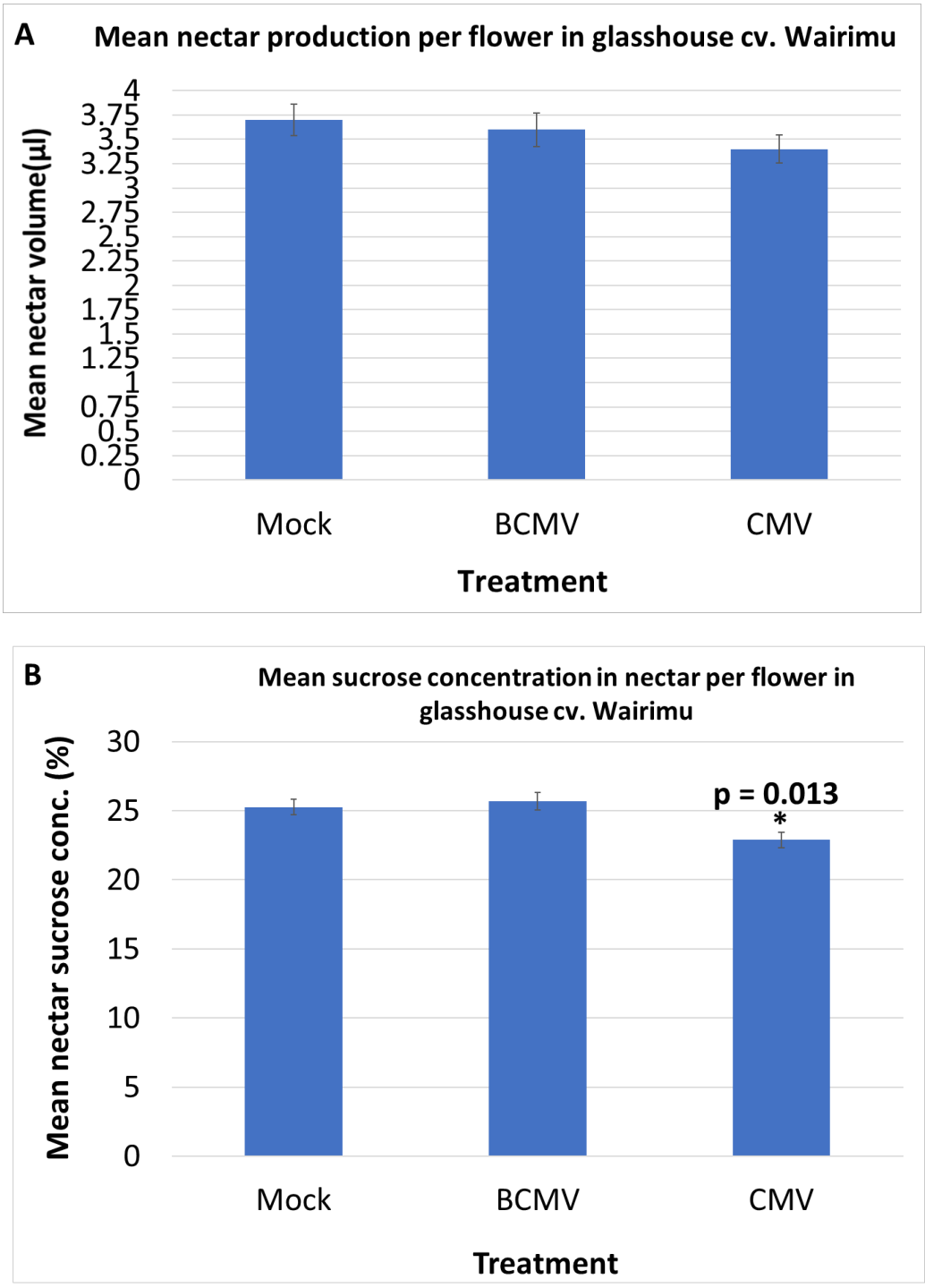


Figure 3.10 Effects of BCMV and CMV on nectar production in cv. Wairimu flowers under glasshouse conditions. (A) Both viruses did not cause any change in floral nectar quantities as compared to flowers from mock-inoculated plants as determined by one-way ANOVA [F (2, 297) = 0.7029, p = 0.5]. (B) Plants infected with CMV produced flowers with significantly lower sucrose

concentration in comparison to mock-inoculated plants [one-way ANOVA: F (2, 297) = 6.6025; p = 0.0016). Error bars represent the standard error of the mean. * Represents significant statistical difference to mock treatment using Tukey's HDS multiple comparisons of means test at 95% family-wise confidence level as indicated by the p-values.

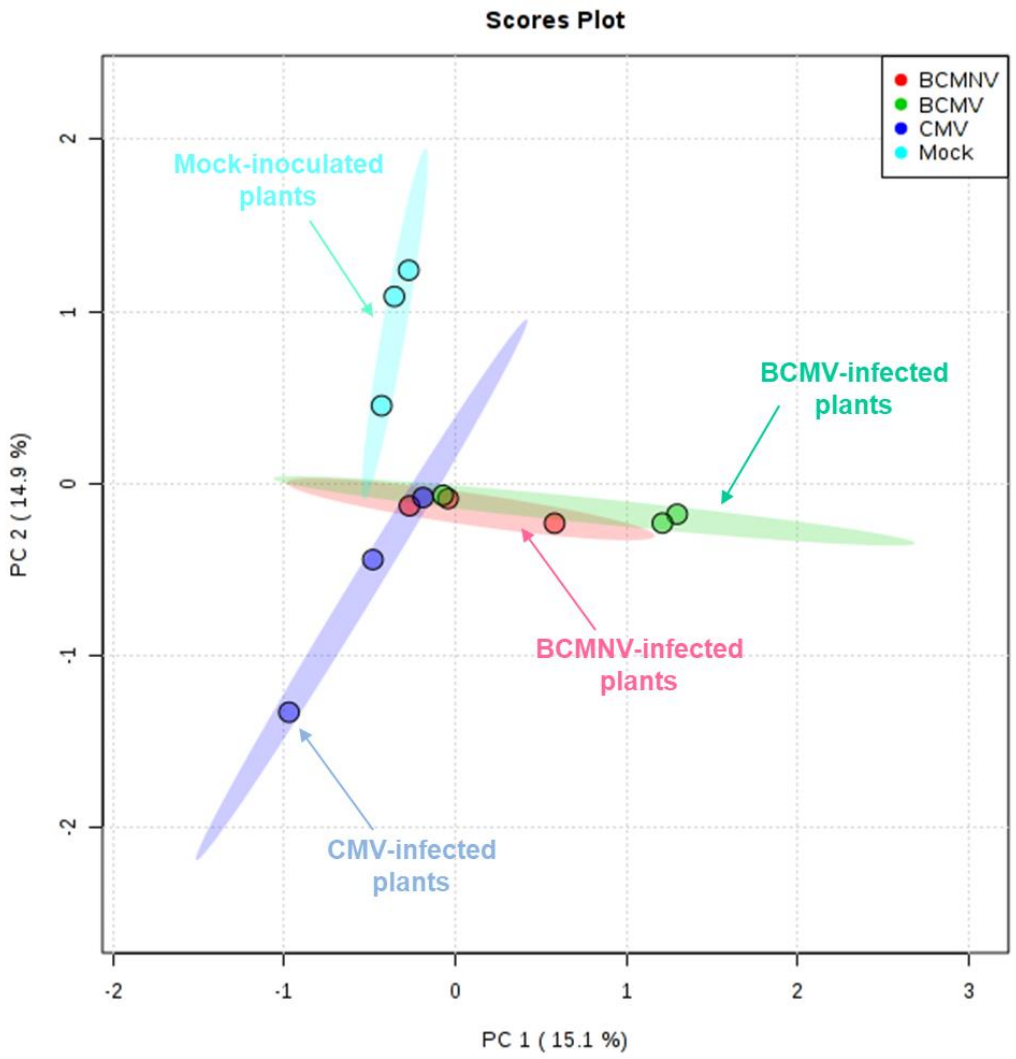
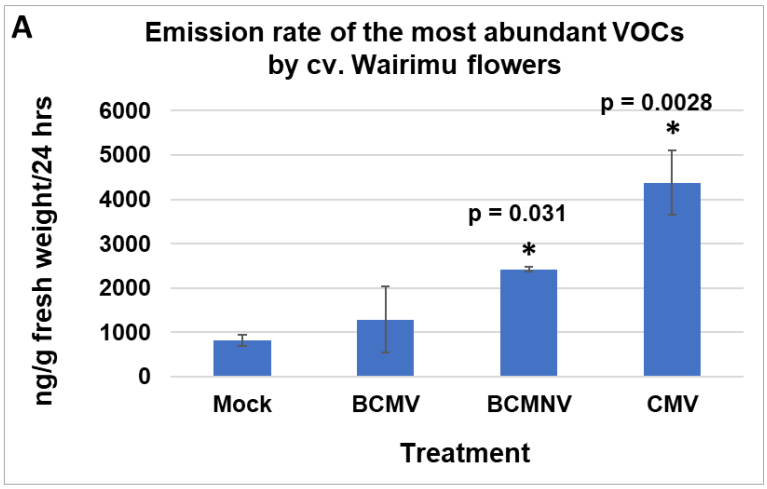


Figure 3.11 Principal component analysis of mass spectrometry data showing clusters of samples based on their similarities. Samples from the same treatment group clustered together showing qualitative differences in VOC blends emitted by mock-inoculated cv. Wairimu flowers (cyan cluster) and virus-infected cv. Wairimu flowers. The cluster ellipses were generated at 95% confidence intervals. PC1 explains 15.1% of the variation and PC2 14.9%. Cumulatively, the variation of the four treatment groups, as explained by PC1 and PC2 is 30%.



List of the most abundant VOCs
Phenol (9.34)
Linalool (RT 9.7)
Unknown VOC (RT 11.98)
Palmitic acid (13.4)
Tetradecane (RT 15.42)

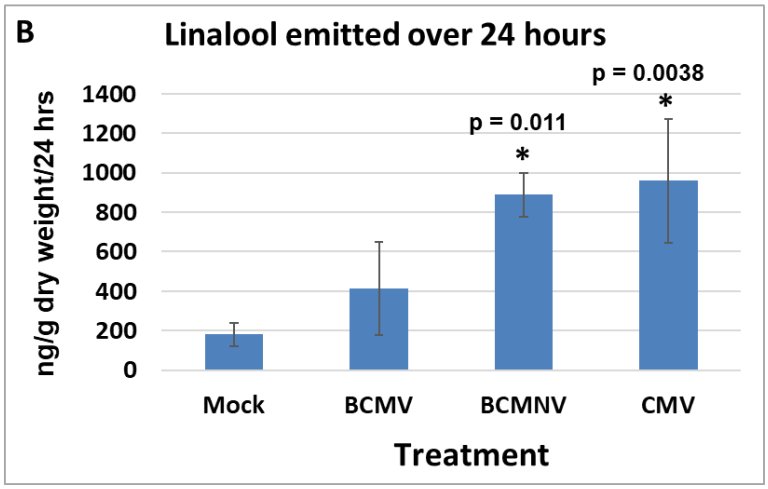


Figure 3.12 Virus-induced quantitative changes in the emission of the six most abundant VOCs emitted by cv. Wairimu flowers. (A) Average estimated quantity of the five most abundant VOC emitted by 8 flowers per treatment of cv. Wairimu plants in 24 hours. BCMNV and CMV-infection induced significantly higher quantities of the VOCs to be emitted (t-tests: t-value = -4.10925 and t = -3.58213 respectively). (B) The emission of linalool was increased from flowers from BCMNV and CMV-infected plants (t-tests: t= -3.60043 and t = -4.58295 respectively). BCMV infection did not induce the production of larger quantities of the above-mentioned VOCs. Error bars represent the standard error of the mean. * Represents significant statistical difference to mock treatment using two-sample t-tests comparisons of independent means at 95% confidence level.

Table 3.4 List of VOCs produced by flowers of cv. Wairimu plants across all treatments (Mock, BCMNV, BCMV and CMV-infected plants). VOCs were collected from fresh cut flowers over 24 hours, eluted in diethyl-ether and analysed with GC-MS (Section 2.6). Identities of VOCs were confirmed by comparing the spectra with those in the NIST spectral databases.

Retention time	Predicted compound	Compound type
6.57	Unknown	Unknown
6.71	2-Methoxy-1,3-dioxolane	Miscellaneous compound
8.25	Isopropyl acetate	Ester
8.49	Hexane	Alkane
9.01	1-ethyl-3-methylbenzene	Aromatic hydrocarbon
9.07	Decane	Alkane
9.17	1-Tridecene	Alkene
9.34	Phenol	Alcohol
9.7	Linalool	Monoterpene
9.74	Dodecanal	Aldehyde
9.86	Dodecane	Alkane
9.94	2-ethyl-1-hexanol	Alcohol
9.96	Isooctanol	Aliphatic compound
10.29	Decane	Alkane
10.42	Octanal	Aldehyde
10.52	Oleyl alcohol, trofluorocetate	Alcohol
10.61	3-butylcyclohexananone,	Ketone
10.79	Undecane	Alkane
10.83	Nonanal	Aldehyde
11.23	Ethyl iso-allocholate	Steroid
11.47	1-Tridecene	Alkene
11.74	Naphthalene	Aromatic hydrocarbon
11.79	Ethylene oxide	Ether
11.86	1-Decanol	Alcohol
11.98	Unknown	Unknown

12.13	n-Tridecanol	Alcohol
12.42	p-Pentylacetophenone	Aromatic hydrocarbon
12.83	Hexadecane	Ether
12.85	Ethylidene-1H-indene	Aromatic hydrocarbon
13.07	Siloxane	Organosilicon *
13.4	Palmitic acid	Fatty acid
13.53	(1-Hydroxy-2,4,4-trimethylpentan-3-yl) 2-methylpropanoate	Ester
13.55	α-Carotene	Carotenoid
13.73	Tetradecane	Alkane
13.82	Diphenyl ether	Ketone
14.39	Isoshyobunone	Ketone
14.75	Butylated hydroxytoluene	Phenol
14.84	Dibenzofuran	Ether
15.1	Geranyl isovalerate	Ester
15.42	Tetradecane	Ester
16.51	Eicosane	Alkane
17.16	1-Chloro-octadecane	Alkane

* Likely contaminant from equipment.

flowers of BCMNV and CMV-infected plants (Figure 3.12B). Forty-three VOCs were emitted by flowers of cv. Wairimu plants across all treatments but in varying quantities. They included different compound groups of esters, alkanes, aromatic hydrocarbons, alcohols, monoterpenes, aliphatic compounds, aldehydes, ketones, steroids, ethers, acids and carotenoids. These are listed in Table 3.4. The most abundant group was alkanes. Two of the compounds could not be identified, and one was identified as a contaminant, siloxane, probably derived from the column (Dr J. Caulfield, pers. comm.).

3.3 DISCUSSION

3.3.1 Delays in flowering induced by BCMV and BCMNV infection may confer a reproductive advantage on plant hosts

I observed a delay in the onset of flowering of 2-5 days in BCMV and 8 to 11 days in BCMNV-infected plants. Onset of flowering can either be advanced or delayed in some virus-infected plants. Groen *et al*. (2016) observed earlier flowering in CMV-infected tomato plants, whereas flowering was delayed by 10 days in tobacco infected with “severe etch virus” (a synonym for the potyvirus tobacco etch virus) (Stein 1962; Shepherd & Purcifull 1971). Alexander *et al*. (2017) observed a delay in flowering in *Panicum virgatum* L. (switchgrass) infected with Barley yellow dwarf virus. Onset of flowering is regulated by abiotic factors such as photoperiod and vernalisation, as well as by gibberellic acid (GA) hormone (Phillips 1998; Wigge 2011). GA also promotes stem elongation and induces seed germination (Sun & Gubler 2004). Helliwell and colleagues (1998) showed that ent-kaurene oxidase is a key factor in the biosynthesis of gibberellins. Zhu *et al*. (2005) showed that ent-kaurene oxidase interacts with the P2 outer capsid protein of *Rice dwarf virus* (RDV). RDV induced a dwarf phenotype in rice plants and reduced levels of ent-kaurene oxidase and GA1, but exogenous application of GA3 rescued these defects (Zhu *et al*. 2005). TuMV infection of nonheading Chinese cabbage decreased GA accumulation

Table 3.5 Summary of results of floral traits in cv. Wairimu induced by viruses in comparison to mock treatment.

Treatment	Flower morphology	Flower colour and patterning	Epidermal cell morphology	VOCs	Nectar reward	
					Volume	Sucrose concentration
BCMV	No change	Change perceivable by bees	No change induced	Qualitatively different, but not quantitatively different from Mock	Increased under controlled conditions, no change in glasshouse conditions	Increased under controlled conditions, no change in glasshouse conditions
BCMNV	No change	Change perceivable by bees	No change induced	Qualitatively and quantitatively different from mock	No change under controlled conditions	No change under controlled conditions
CMV	No change	Change perceivable by bees	No change induced	Qualitatively and quantitatively different from mock	No change under controlled and glasshouse conditions	Increased under controlled conditions and decreased in glasshouse conditions

(Wang *et al*. 2011). Speculatively BCMNV being a virulent virus that causes severe symptoms in hosts, may interfere with GA signalling pathway resulting in a delay in flowering in common bean.

To reduce competition for pollinators, some plants have evolved ways of altering flowering time (Frankie 1975; Anderson & Schelfhout 1980; Berrached *et al*. 2017). It has been shown that a delay or advance in flowering may have a pollination advantage over on-time flowering because of increased intensity of pollinator visitation (Eberle *et al*. 2014). Eberle *et al*. (2014) observed a delay in the onset of flowering but increased intensity of visitation by pollinators in *Echium plantagineum* L., which they sow early in summer (late-sown treatment). The delay in onset of flowering in particularly BCMNV-infected plants might provide an incidental benefit for hosts by decreasing competition for a limited number of pollinators. Should this occur in natural ecosystems, susceptible hosts may have an advantage of avoiding competition over uninfected or resistant plants since pollination service is limited. However, this school of thought is argued against by other authors who suggest that early or late flowering might result in flowers being produced before pollinators are available or after pollinators are available, respectively (reviewed in Mitchell *et al*. 2009b).

Reproductive isolation is another possible outcome that might result from late flowering of virus-infected plants. Thus, if there is no overlapping of flowering times between healthy uninfected plants and BCMNV-infected plants, these two groups of plants will reproduce in isolation, and over repeated generations, two distinct populations of virus-resistant and virus susceptible populations will be resultant as illustrated in Figure 3.13A. However, I observed an overlapping flowering period between mock-inoculated and BCMNV-infected plants, despite the delay in flowering. I propose that the overlapping flowering period will allow cross-pollination between the two groups and passaging of alleles of BCMNV-susceptibility onto the next generations (Figure 3.13B). The longer the synchronisation period, the greater the chances of cross-pollination and geneflow. The

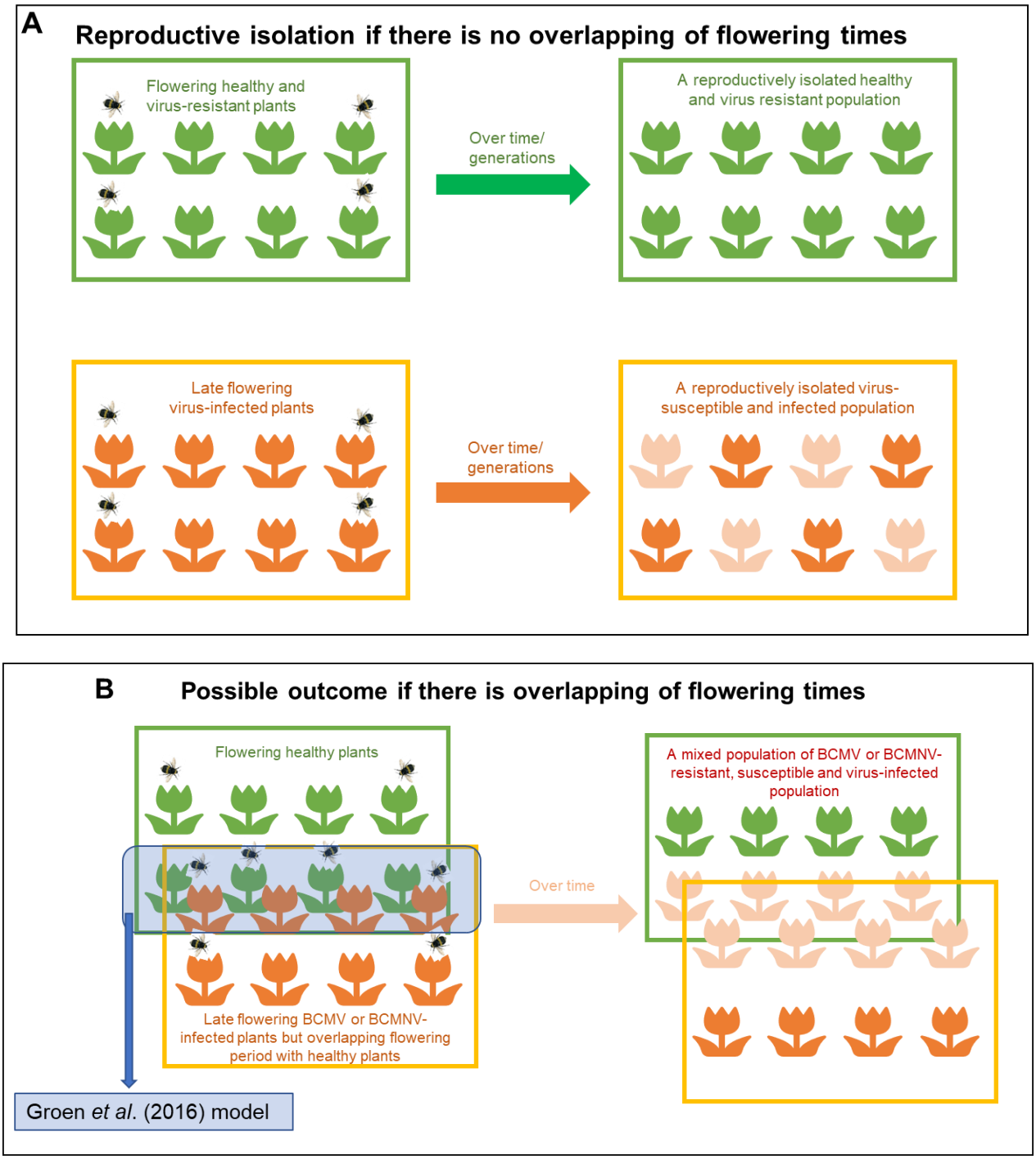


Figure 3.13 Schematic diagrams representing possible outcomes of isolated flowering and synchronised flowering regimes. In (A), flowering isolation is represented whereby virus-infected plants and health/uninfected/resistant plants flower at different times. This will lead to reproductive isolation of healthy and virus-infected plant populations. Resultant populations over

generations will be distinct because there is no gene flow between the two groups of plants in sympatry. In (B), BCMNV-infected plants and mock-inoculated plants flowering regimes are represented, where there is overlapping of flowering period. Cross-pollination and gene flow facilitated by bees will result in a mosaic population that will consist of healthy/resistant plants, BCMNV-susceptible plants and BCMNV-infected plants.

resulting populations will support the mathematical evolutionary model by Groen *et al*. 2016, of which BCMV-infected plants fit into the model to a greater extent than BCMNV-infected plants.

Gross & Werner (1983) contended that synchronization of flowering increases pollinator visitation and is the key to successful seed setting and maximum cross-pollination (Augspurger 1983; Medan & Bartoloni 1998). In my experiments, CMV-infected plants flowered at the same time as mock-inoculated plants, which may lead to greater chances of being cross-pollinated because of increased pollinator visitations to the site. I propose that flowering synchronization between mock-inoculated and CMV-infected plants in the presence of pollinators will promote gene flow and hence passaging of alleles of susceptibility in next generations (Figure 3.14), as proposed in the Groen *et al*. (2016) mathematical evolutionary model. Over generations, CMV-susceptible plants may predominate over healthy/resistant plants. Contrastingly, flowering synchrony may result in a shortage of possible pollinators and competition among plants for visits (reviewed in Mitchell *et al*. 2009b). This effect might work to the disadvantage of CMV-infected and mock-inoculated plants that flower at the same time.

3.3.2 Virus infection reduces flower size and number

Virus-infected cv. Wairimu plants had fewer flowers than mock-inoculated plants. The direct effect of decreased flower numbers is yield reduction (Williamson & Miller 2002) and may help explain why BCMV, BCMNV and CMV infections cause significant crop losses (reviews by Morales *et al*. 2006; Jacquemond 2012; Worrall *et al*. 2015). It has been shown that plants with more flowers may attract more pollinators than plants with few flowers (Klinkhamer & de Jong 1990; Mitchell 1994; Galloway *et al*. 2002). However, it has been shown that large floral display does not always increase visitation rates per plant because pollinators may probe a smaller proportion of flowers on large displays, as observed in *Myosotis* spp. and *Mimulus* spp. (Robertson & Macnair 1995), *Symphytum officinale* L. (common comfrey) (Goulson *et al*. 1998), *Cynoglossum officinale* L.

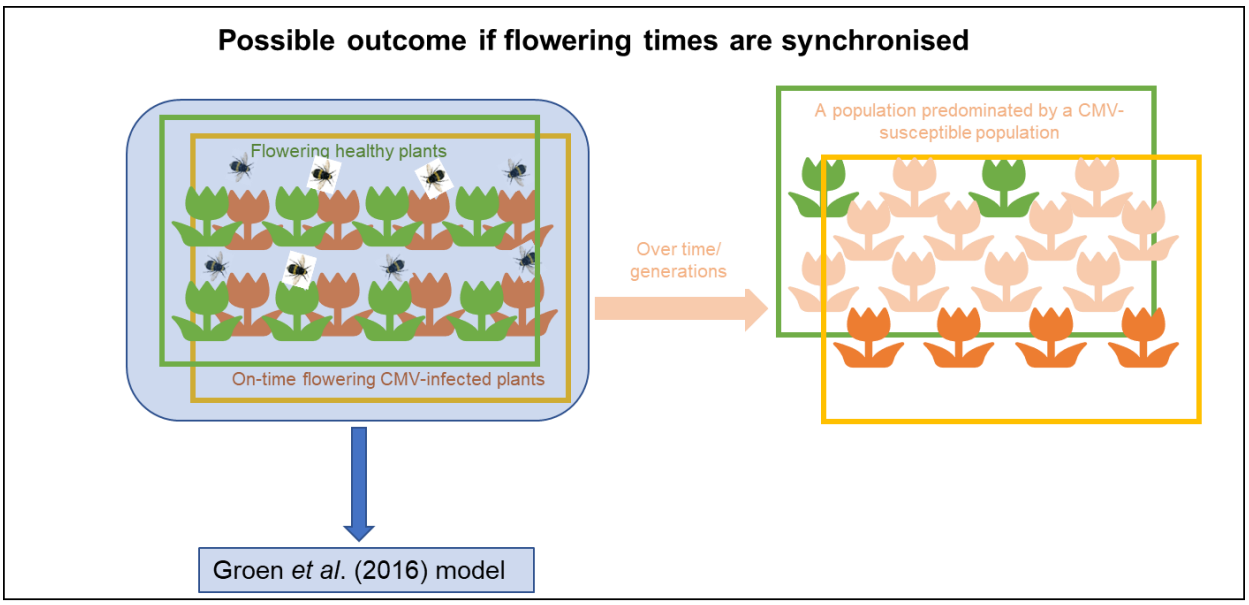


Figure 3.14 A schematic diagram of synchronisation of flowering times in mock-inoculated and CMV-infected plants. Pollinators will facilitate cross-pollination between healthy/uninfected plants and CMV-infected plants, resulting in a population dominated by CMV-susceptible plants over generations.

(houndstongue) (Vrieling *et al*. 1999), and *Mimulus ringens* L. (square-stemmed monkeyflower) (Mitchell *et al*. 2004).

Petals of flowers produced by virus-infected plants were slightly smaller (especially those from CMV-infected plants) than flowers from mock-inoculated plants. This trend was consistently observed, but differences were not statistically significant. Virus infections are known to reduce flower sizes, for example in CMV infection in *Centranthus ruber* L. (spur valerian) (Carrieri *et al*. 2012) and for five colour-breaking potyviruses in tulip and lily (Dekker *et al*. 1993). Reduced flower size induced by virus infection may disadvantage virus-infected plants from pollination by bees that are more attracted to large flowers because they are more conspicuous from a distance (Goulson 1999; Duffield *et al*. 2008). Moreover, there is a correlation between the size and reward of flowers in some species, and hence preference for large flowers in these species may be because bees associate bigger flowers with better rewards (Stanton & Preston 1988; Ashman & Stanton 1991; Fenster *et al*. 2006). However, pollinators with short tongues, like hoverflies and beetles, are attracted to smaller flowers because it is easier for them to access pollen and nectar (Colley & Luna 2000). In common bean BCMV, BCMNV and CMV did not significantly reduce flower sizes, hence infected plants are most likely to be visited by the same pollinators as healthy plants (either with long or short tongues) in natural environments.

3.3.3 Viruses cause colour changes on nectar guides that may be perceptible to bees

Wing petals from BCMV, BCMNV and CMV-infected cv. Wairimu plants appear the same to bees as those from mock-inoculated plants as shown by the same position they occupied in the bee-colour hexagon. The nectar guides of standard petals of BCMV, BCMNV and CMV-infected common bean plants showed marked variations (no statistical testing done) in colours compared to those from mock-inoculated plants (Table 3.5). These colour variations are likely to be

perceptible to bees. The human eye cannot distinguish these colour changes in the ultraviolet spectrum induced by virus infection (Penny 1983).

Positions occupied by each mean standard nectar guide in the hexagon varied by more than 0.008 hexagon units (Figure 3.7 & Table 3.2). Honey bees have been shown to discriminate between colours as little as 0.008 hexagon units apart with 75% accuracy (Dyer & Neumeyer 2005). However, bumblebees' discrimination seems to be poorer than that of honey bees; the same colour as above is discriminated with less than 60% accuracy (Dyer *et al*. 2008). Bumblebees poorly distinguish between colours of less than 0.07 hexagon units apart (Dyer *et al*. 2006). Thus, the difference between each position is more than enough for honey bees to perceive the differences in colour between uninfected and virus-infected plants but insufficient for bumblebees.

Nectar guides have been shown to significantly decrease flower handing and reward search time in bumblebees (Waser & Price 1985; Leonard & Papaj 2011). They have also been shown to reduce chances of nectar robbing by bumblebees because they induce bumblebees to collect nectar legitimately more frequently (Leonard *et al*. 2013). It is not known whether the slight colour changes induced by virus-infection on nectar guides in common bean will reduce flower search and handling time by bees. I could not test the response of laboratory colonies of bumblebees to determine if there would be any preference or effect on visitation times for the colour differences identified because of time constraints.

3.3.4 Viruses do not alter the morphology of conical cells on petal epidermal layer of cv. Wairimu flowers

Striated conical cells on the abaxial side of wing petals had similar morphology in flowers of both virus-infected plants and mock-inoculated common bean plants. Generally, conical cells are only found on the adaxial surface of petals (Kay *et al*. 1981; Christensen & Hansen, 1998), which is the petal surface that most pollinators interact with. However, the zygomorphic structure of legume

flowers means that the abaxial surface of wing petals contacted by pollinators when visiting the flowers (Stoddard 1991). Conical cells hence play an essential role in the tripping mechanism of common bean. Bees have been shown to have a strong preference for flowers with conical epidermal cells (Whitney *et al*. 2009; Alcorn *et al*. 2012) because bees use less energy when handling the flower. Flat surfaces would mean the bees would slip and hence would need to continuously flap their wings to stay on the flower. On that note, both virus-infected and mock-inoculated plants offer the same energy-saving advantage to bees with their conical cells which are similar in morphology.

3.3.5 Viruses increase nectar reward in common bean

Flowers from BCMV-infected cv. Wairimu plants produced larger quantities of nectar and nectar from flowers of both BCMV and CMV-infected plants had higher sucrose concentrations than flowers from mock-inoculated plants when grown in controlled conditions (Table 3.5). Bees have a limited diet of pollen and nectar, nectar being a source of energy for the queen and workers and pollen supplies protein for developing larvae, freshly emerged workers and the queen (Haydak 1970; Alford 1975; Crailsheim 1992; Roulston & Cane 2000). For this reason, bees innately prefer better-rewarding flowers. Several studies have shown that bees are deterred by low reward encounters (Real 1981; Waddington *et al*. 1981; Shafir *et al.* 1999) showing strong preferences for flowers with the greatest volume of nectar containing the highest sucrose concentration (Cnaani *et al*. 2006; Konzmann & Lunau 2014). However, when both variables are altered, higher sucrose contents are preferred compared to volume, even when the net energy reward is smaller (Cnaani *et al*. 2006).

The likelihood of a bee returning to plants of a particular species depends on the quality and quantity of the rewards because it can learn to effectively associate specific floral features with better dietary rewards (Dobson & Bergstrom 2000; Raine *et al*. 2006; Howell & Alarcon 2007;

Gomez *et al*. 2008). In a study by Raine *et al*. (2006), laboratory-reared *B. terrestris audax* were presented with blue non-rewarding and yellow-rewarding artificial flowers. The bees initially preferred nonrewarding blue flowers, but their preference changed after probing rewarding yellow flowers (Raine *et al*. 2006). Raine *et al*. (2006) explained the initial innate preference for blue flowers based on the assumption that blue flowers are generally profitable to *B. terrestris audax* in the British foraging environment as evidenced by preference for blue flowers in all bumblebee species (Chittka & Wells 2004). Thus, increased nectar reward may give a competitive advantage to virus-infected plants over uninfected plants.

However, when plants were grown in glasshouse conditions where they were grown in bigger pots and receiving more light (minimum of 685.5 µmol/m²/s) (see Section 2.2.1.2), flowers from both mock-inoculate and virus-infected plants produced the same quantities of nectar. Flowers from CMV-infected plants produced nectar with significantly less sucrose concentration. Radhika *et al*. (2010) demonstrated that in lima bean (*Phaseolus lunatus* L.), the response to jasmonates as well as isoleucine–jasmonic acid conjugate biosynthesis, which controls the subsequent secretion of extrafloral nectar, is modulated by the ratio of red to far-red radiation. Thus, in the dark, jasmonic acid reduced extrafloral secretion, whereas, under light conditions, jasmonic acid-induced extrafloral secretion relative to controls (Radhika *et al.* 2010). The effect of ambient light conditions on floral nectar production in *P. vulgaris* still remains unknown.

Viruses are known to condition their hosts to perform better under stressful conditions, hence the suggestion of perceiving them as conditional mutualists (Roossinck 2011). Viruses like the *Brome mosaic virus* (BMV) and CMV induced drought resistance in rice and beet, respectively (Xu *et al*. 2008). CMV also induced drought resistance in Arabidopsis (Westwood *et al*. 2013b) and cold tolerance in beet plants (Xu *et al.* 2008). I propose that the production of better rewarding nectar induced by virus infection in common bean cv. Wairimu under poor light conditions (200

µmol/m²/s) as observed in controlled conditions is a conditional payback, thereby making BCMV and CMV-infected plants more appealing to bee pollinators.

It is arguable that nectar production changes observed might be a result of plant response to presence of viruses. Plants respond to herbivory for example, by directly producing toxic chemicals (such as terpenoids, alkaloids, anthocyanins, phenols, and quinones) that either kill or retard the development of or kill the herbivores (Hanley *et al*. 2007). Indirect responses include emission of VOC blends that recruit natural enemies of the herbivores and/or by providing food (e.g. extrafloral nectar) and housing to enhance effectiveness of the natural enemies (Arimura *et al*. 2009). In this view, the production of more nectar rich in sucrose may be perceived as a plant response mechanism to provide food for pollinators to promote reproduction through cross-pollination in the presence of viral pathogens.

Plants have defence mechanisms against viruses that include RNA silencing, SA-mediated defences, and signalling pathways controlled by various plant hormones [jasmonic acid (JA), ethylene (Et), abscisic acid (ABA)] that influence plant antiviral responses (reviewed in Robert-Seilaniantz *et al*. 2011; Alazem & Lin 2015; Carr *et al*. 2019). Multiple levels of crosstalk among RNA silencing and the ABA-, JA-, SA- signalling pathways highlight complex regulatory mechanisms of host plant defence responses that are manipulated by viruses to their advantage. For example, some VSRs , including the cucumoviral 2b protein and the poytviral P1/HC-Pro, interfere not only with antiviral RNA silencing but also with the JA-, Et-, or SA-pathways, in some cases down-regulating plant defence responses to promote their transmission by insect vectors (Ji & Ding 2001; Geri 2004; Westwood 2014; Wu *et al*. 2017; Poque *et al*. 2018). CMV 2b protein was also shown to respectively, interfere and induce with biosynthesis and emission of bee-attracting VOCs in tomato (Groen *et al*. 2016). This was proven by replacing CMV with CMVΔ2b, a mutant unable to express the 2b protein in free choice assays and the mutant could not to induce

any changes in the emission of bee- attracting VOC blends (Groen *et al*. 2016). Ziebell *et al*. (2011) found that infection of tobacco with CMVΔ2b induced strong resistance to *Myzus persicae* while wild type CMV infection fostered aphid survival. Lewsey *et al*. (2010) showed that the 2b protein interferes with the JA signalling pathway, which is important in the induction of resistance to insect herbivores in Arabidopsis.

It is not just VSRs that influence aphid behaviour and fitness. Westwood *et al*. (2014) showed that while a general property of all VSRs is to interfere with the JA signalling pathway, this does not always have a positive effect on aphid fitness suggesting a role for other viral proteins. Casteel *et al*. (2014) transiently expressed different TuMV proteins using transgenic *Nicotiana benthamiana,* and Arabidopsis reported that the fecundity of *M*. *persicae* was significantly increased by NIa-Pro expression but was significantly decreased by the expression of HC-Pro, 6K1, and VPg. Taken together, I propose that viruses induce changes in nectar production, rather than plants responding to presence of viruses. Future work could further investigate which specific viral proteins are responsible for nectar production changes in susceptible hosts. I suspect that CMV 2b and BCMV P1/HC-Pro proteins are responsible, so mutant viruses unable to express these proteins could be used in similar experiments. If the mutant viruses induce no changes in nectar sucrose concentration under low light conditions in cv. Wairimu, these proteins can then be implicated to be the cause of changes observed.

3.3.6 Virus infection alters VOCs blends emitted by common bean flowers

Floral scent plays a vital role in plant-pollinator interactions. VOCs emitted by flowers can trigger landing by bees on flowers (Lunau 1992) and are also equally crucial in long distance advertisement of flowers (Kunze & Gumbert 2001; Raguso & Willis 2002; Chittka & Raine 2006; Dötterl & Schäffler 2007; Burger *et al*. 2010; Glover 2011; Suchet *et al*. 2011). I have shown that virus infection alters the quantitative and qualitative emissions of floral VOC blends in cv. Wairimu

flowers (Table 3.5). Flowers from BCMNV and CMV-infected plants produced larger quantities of five most abundant VOCs in comparison to those from mock-inoculated plants.

VOC blends are more important for insect host plant preference than single compounds. Workers of yellow-faced bumblebees (*B. vosnesenskii* Radoszkowski) preferred a combination of limonene, myrcene and ocimene over any single compound alone (Byers *et al*. 2014). Experiments with *Aphis fabae* aphids on *Vicia faba* showed that aphids use VOC blends, not individual semiochemicals, for host location (Webster *et al*. 2008). When individual volatiles were presented to aphids, all compounds were repellent (Webster *et al*. 2008). However, when the VOCs were blended at their most repellent concentrations, they became attractive to *A. fabae* (Webster *et al*. 2010). However, the blends differ depending on quantities of individual compounds.

Viruses can either increase or decrease the emission of certain individual compounds as reported in Groen *et al.* (2016), and the resultant blends can be more attractive to naïve bees as seen in CMV-infected tomato and bumblebees (Groen *et al*. 2016). It has been shown that potato leaf roll virus induced changes in the VOC profiles of potato, and this caused *Myzus persicae* aphid attraction to the plant even in the absence of visual stimuli (Eigenbrode *et al*. 2002). In current this study, linalool emission by flowers from BCMNV and CMV-infected plants was increased compared to BCMV-infected and mock-inoculated plant flowers. Linalool is one of the two most widespread compounds among floral scents (Knudsen *et al*. 2006). It is an attractant for various diurnal bees (Dötterl & Vereecken 2010), nocturnal bees (Krug *et al*. 2018) and for other nocturnal visitors, such as moths or bats (Dobson 2006). Producing larger quantities of linalool may attract more bees and hence BCMNV, and CMV-infected plants might have a competitive advantage over mock-inoculated plants.

Lederberg (2000) mentioned that “microbes have a shared interest in their host’s survival: a dead host is a dead-end for most invaders too.” Thus, pathogens may have evolved in ways that have happened to ensure the survival of susceptible hosts and in this way, prevented their own extinction. In this chapter, I have shown that BCMV and BCMNV delay flowering time, all the three viruses induce changes in nectar guide colour as perceived by bees, BCMV increases nectar production and BCMV and CMV increase nectar sucrose concentration under certain conditions and viruses infection induce changes in VOCs blend emission in cv. Wairimu plants. The observed changes in floral traits induced by virus infections in common bean may correspond to the need to reduce competition for pollinators and/or to be more competitive over healthy plants, a phenomenon that has been observed in flowering plants. Some of these changes were tested in work described in subsequent chapters to determine if the ‘payback’ hypothesis holds under controlled and natural environments.

CHAPTER 4

VIRUSES INDUCE CHANGES IN VOLATILE ORGANIC COMPOUND EMISSION PROFILES IN COMMON BEAN

4.1 INTRODUCTION

The sessile “life-style” of plants led to the evolution of strategies like emission of VOCs to function as signals in plant-plant communication, defence against herbivores and pathogens, and to attract pollinators, seed dispersers and other beneficial organisms (Dudareva & Pichersky 2008). Bees have a poor visual resolution (Chittka & Raine 2006) and hence also rely on olfactory signals when foraging and pollinating flowers. VOCs can trigger an immediate decision to land on a flower when the pollinator is close by (Lunau 1992). Viruses induce changes in emitted VOC blends emissions in host plants, as well as other metabolic reactions, that can affect interactions with pollinators, such as bumblebees (Groen *et al*. 2016; Jiang 2017) and attract or repel virus vectors such as aphids (Fereres & Moreno 2009; Mauck *et al*. 2010; 2012; Westwood *et al*. 2013a; Tungadi *et al*. 2017; Carr *et al*. 2018; Wamonje *et al*. 2019) (see section 1.5).

In this chapter, I investigated if BCMNV, BCMV or CMV infection induces qualitative and quantitative changes in VOC emission by non-flowering and flowering common bean plants. The VOCs were collected for 24 hours by dynamic headspace trapping (Section 2.6.1), and separation of eluted VOCs was done using GC-MS (Section 2.6.2). Plant material (leaves from non-flowering plants and leaves plus flowers from flowering plants) fresh weight and dry weight were measured to normalize the VOC abundance. Principal component analysis on the mass spectra was performed with MetaboAnalyst 4.0 and identities of VOCs were confirmed by comparing the spectra with those in the NIST spectral databases (see Section 2.6.2). Only a few VOCs that are electrophysiologically active and known to attract bees were quantified. Bee-attracting VOCs that were quantified are limonene, linalool, ocimene, pinene and benzaldehyde (Granero *et al*. 2005;

Knudsen *et al*. 2006; Dötterl & Vereecken, 2010; Klatt *et al*. 2013; Krug *et al*. 2018). According to Klatt *et al*. (2013), benzaldehyde, (z)-3-hexen-1-ol acetate, D/L-limonene and nonanal were electrophysiologically active VOCs on antennae of red mason bees (*Osmia bicornis* Linnaeus), and these volatiles were also quantified in this study. VOCs that were electrophysiologically active in electroantennography assays on black bean aphid (*Aphis fabae*) antennae in Wamonje *et al*. (2019) quantified in this study are (z)-3-hexen-1-ol acetate, nonanal and (E)-8-dimethyl-1,3,7-nonatriene (DMNT). Benzaldehyde, linalool, ocimene and pinene were quantified using standard curves of pure benzaldehyde, linalool, ocimene, and pinene, respectively. All the other most abundant VOCs were quantified using pinene equivalent standards. A multivariate analysis of variance (MANOVA) was done in cv. Dubelle witte market class B and in cv. Wairimu because these had more than two independent variables (treatment groups) and two or more dependent variables (volatiles). MANOVA could not be done in cv. Dubelle witte market class A because I only had two independent variables (mock and CMV treatments). This was done because multiple testing assumes a cause-effect relationship whereby one or more independent, controlled variables (the factors) cause a significant difference in one or more characteristics. The reason being that biosynthesis pathways of VOCs in plants interfere with each other. However, MANOVA could not show whether virus treatments caused significant changes in VOC quantities in comparison to mock treatment, which was the main question to be answered in this section. Hence ANOVA and post-hoc tests were done to answer the objectives of the project.

4.2 RESULTS

4.2.1 Viruses induce qualitative and quantitative changes in VOCs emitted by *P. vulgaris*

VOCs were collected from non-flowering and flowering cv. Dubbele witte market classes A and B and flowering cv. Wairimu plants. Dubbele witte market class A was used for VOC experiments

with CMV, whereas Dubbele witte market class B was used in experiments with BCMV and BCMNV.

In Dubbele witte market class A, GC-MS results examined by PCA revealed overall qualitative changes in the volatile blends of both flowering and non-flowering CMV-infected plants. The VOC blends from mock-inoculated plants clustered separately from those of the CMV-infected plants (Figure 4.1). Thus, the emitted VOCs were distinct from each other when compared by the relative intensity of ions (over 80 Da in size) within the samples. In non-flowering plants, 33 VOCs were detected, and 28 of them were identified. These consisted of three alcohols, five aldehydes, one aliphatic amine, one alkene, four aromatic hydrocarbons, four esters, two ethers, seven hydrocarbons, one ketone, and one steroid (Table 4.1). The GC-MS analysis of the samples showed that CMV-infected and mock-inoculated plants shared some similar VOCs but with differences in quantities of emissions between non-flowering and flowering plants (Figures 4.2, 4.3 & 4.4). Visual analysis of the peak areas showed that some VOCs appeared to be increased in CMV-infected plants in comparison to those of mock-inoculated plants, although not statistically tested. These were; nonane (RT) 8.14); decane (RT 9.11 and 9.65); 1,1-bis(dodecyloxy)hexadecane, (RT 9.24); dodecane (RT 9.94); lauraldehyde (RT 10.38); undecane (RT 10.88); and an un-identified VOC (RT 13.17). There were VOCs that appeared to be less abundant because of CMV infection in comparison to those from mock-inoculated plants. These were ethyl iso-allocholate (RT 11.45 & 13.78) and 1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate (RT 15.54) (Figure 4.2).

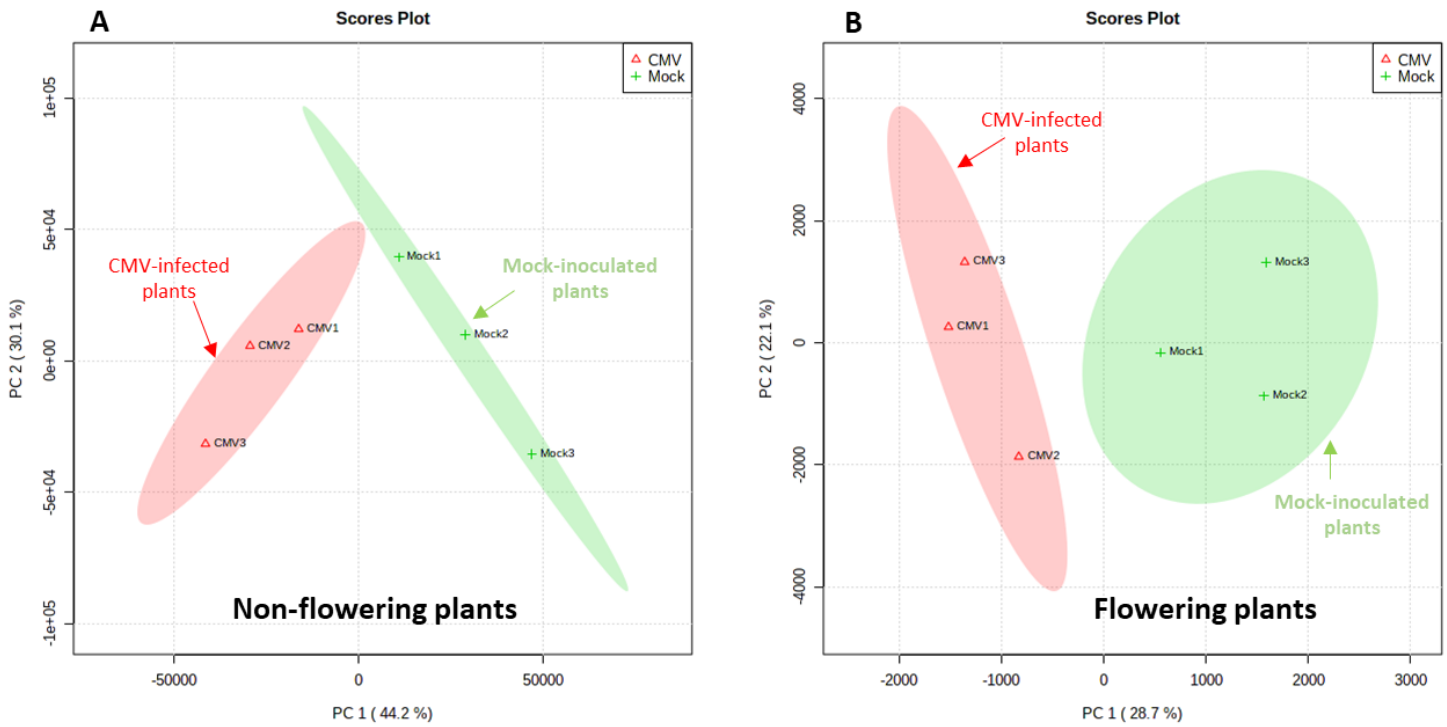


Figure 4.1 PCA of m/z values (binned to 1.0 Da) obtained by gas chromatography-mass spectrometry of samples of VOCs from Dubbele witte bean market class A (A) non-flowering and (B) flowering plants.

The VOCs were collected by dynamic headspace trapping from mock-inoculated (green), and CMV-infected (red) plants. The analyses show discrimination between the two treatments both in non-flowering and flowering plants. The first and second principal components (PC1 and PC2) accounted for (A) 74.5% and (B) 50.8% of the total variation of the volatiles from the two treatment groups, respectively.

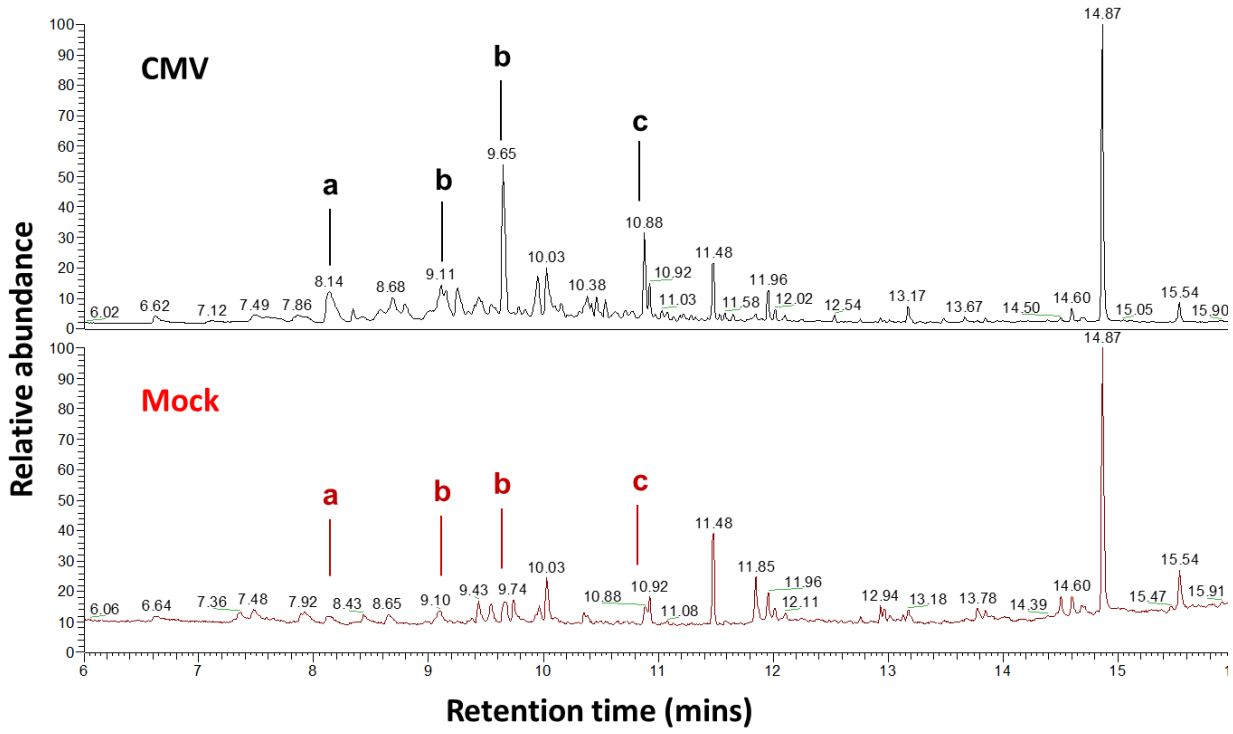


Figure 4.2 Gas chromatograms of VOCs emitted by mock-inoculated (red) and CMV-infected (black) non-flowering Dubbele white market class A. A capillary GC column fitted with a programmable temperature vaporizer injector was directly coupled to a mass spectrometer. Eluted peaks are shown in the gas chromatography graph with their retention times (RT). Several VOCs in CMV-infected plants are produced in larger quantities than in mock-inoculated plants (Mock) as described in section are 4.1. Identities of VOCs and their retention times are listed in Table 4.1. The most abundant VOCs that appeared to be increased in CMV-infected plants are (**a**) nonane (RT 8.14); (**b**) decane (RT 9.11 and 9.65); (**c**) undecane (RT 10.88).

Table 4.1 List of VOCs and their retention times for non-flowering mock-inoculated and CMV-infected Dubbele witte market class A plants.

Retention time (mins)	Predicted compound	Compound type
6.62	Methyl 14-(2-octylcyclopropyl)tetradecanoate	Ester
7.36	Docosahexaenoic acid, 1,2,3-propanetriyl ester	Ester
7.49	1,3-dimethylbenzene	Aromatic hydrocarbon
8.14	Nonane	Hydrocarbon
8.34	sec-Butyl nitrate	Aliphatic anime
8.65	Decane	Hydrocarbon
8.80	1-Tridecene	Alkene
9.06	Benzaldehyde	Aldehyde
9.11	Decane	Hydrocarbon
9.24	1,1-Bis(dodecyloxy)hexadecane	Ether
9.55	1,2,3-Trimethylbenzene	Aromatic hydrocarbon
9.65	Decane	Hydrocarbon
9.74	(E)-3-Hexen-1-ol acetate	Ester
9.94	Dodecane	Hydrocarbon
10.03	Linalool	Alcohol
10.38	Lauraldehyde	Aldehyde
10.88	Undecane	Hydrocarbon
10.92	Nonanal	Aldehyde
11.03	Cyclopentadecanol	Alcohol
11.48	Siloxane	Organosilicon*
11.58	Undecanal	Aldehyde
11.68	Z-9-Hexadecen-1-ol	Alcohol
11.85	Naphthalene	Aromatic hydrocarbon
11.96	Dodecane	Hydrocarbon

12.02	Decanal	Aldehyde
12.54	1,3-bis(1,1-Dimethyl)benzene	Aromatic hydrocarbon
12.94	Unknown	Unknown
13.17	Unknown	Unknown
13.67	Unknown	Unknown
13.78	Ethyl iso-allocholate	Steroid
14.5	Unknown	Unknown
14.6	2,2,7,7-Tetramethyltricyclo[6.2.1.0(1,6)]undec-4-en-3-one	Ketone
14.87	Siloxane (Contaminant)	Organosilicon*
15.54	1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate	Ester

*Likely contaminant from equipment.

The most abundant VOCs were quantified and among them were electrophysiologically active ones (Section 4.1; Figures 4.3 & 4.4) and VOCs known to attract bees (Section 4.1; Figure 4.4 A & B) as stated in Section 4.1. The overall emission of the most abundant VOCs in CMV-infected plants was significantly increased compared to mock-inoculated plants (Figure 4.3). Benzaldehyde and linalool were elevated, whereas nonanal and 3-hexen-1-ol acetate were slightly decreased in CMV-infected plants (Figure 4.4).

In flowering Dubbele witte market class A, 24 VOCs were detected, and 19 of them were identified using the NIST library (Table 4.2). Functional groups of these VOCs are two alcohols, three aldehydes, two aromatic hydrocarbons, one aliphatic amine, four esters, one ether and six hydrocarbons. VOCs that appeared to be relatively more abundant in CMV-infected plants are 1-octen-3-ol (RT 9.37); 4-hexen-1-ol acetate (RT 9.74); D-limonene (RT 10.02); linalool (RT 10.88); nonanal (RT10.93); decanal (RT 12.02) and 1-isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate (RT 15.53) (Figure 4.5 & Table 4.2), although not statistically increased. Changes of decreased VOCsemitted by CMV-infected plants in comparison to those of mock-inoculated plants, although not statistically confirmed, are seen in 3- Hexen-1-ol, (Z)- (RT 7.35); β-ocimene (RT 10.27) and an unknown VOC (RT 13.17) (Figure 4.5 and Table 4.2). The greatest increase in VOC emission quantities in CMV-infected plants when compared to those of mock-inoculated plants were in 4-hexen-1-ol acetate (RT 9.74), D-limonene (RT 10.02) and 1-isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate (RT 15.53) (Figure 4.5 & Table 4.2).

The most abundant and known electrophysiologically active VOCs were quantified as well, including those that are known to attract bees (Section 4.0; Figures 4.6 & 4.7). There was no significant difference in the total emission rate of the most abundant VOCs (Figure 4.6).

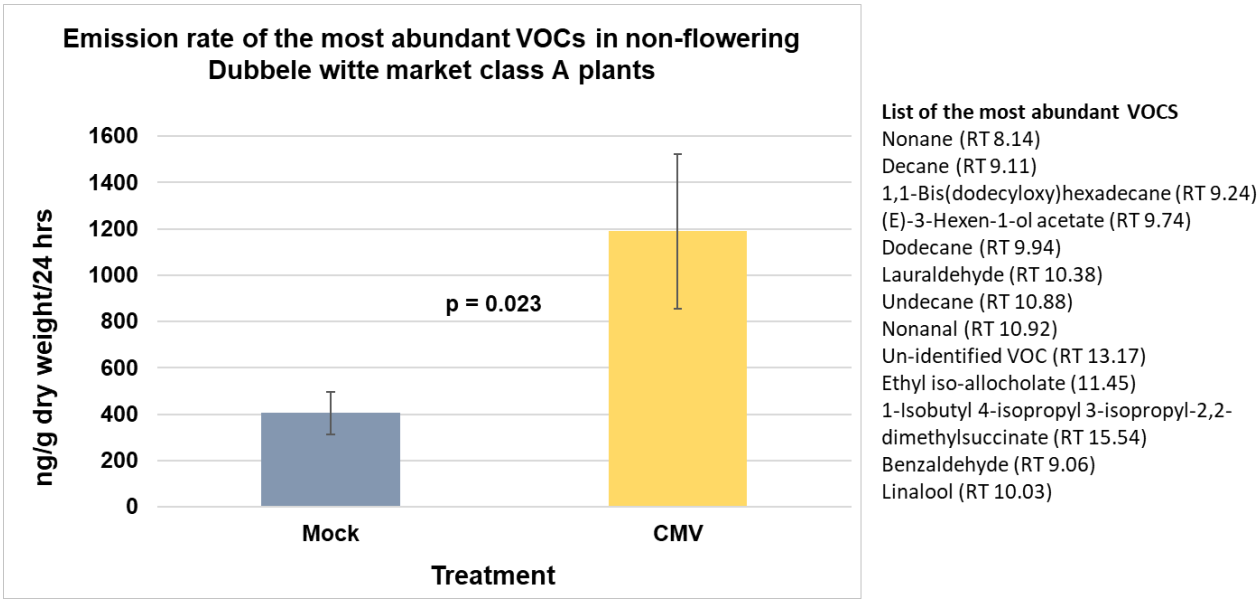


Figure 4.3 Virus-induced quantitative changes in the emission of the most abundant VOCs in cv. Dubbele witte market class A non-flowering plants. Quantification of the most abundant VOCs showed that the emission rate was significantly elevated in CMV-infected plants. The p-value shown is from a two-sample t-test (t = 2.31). Error bars represent standard error of the mean.

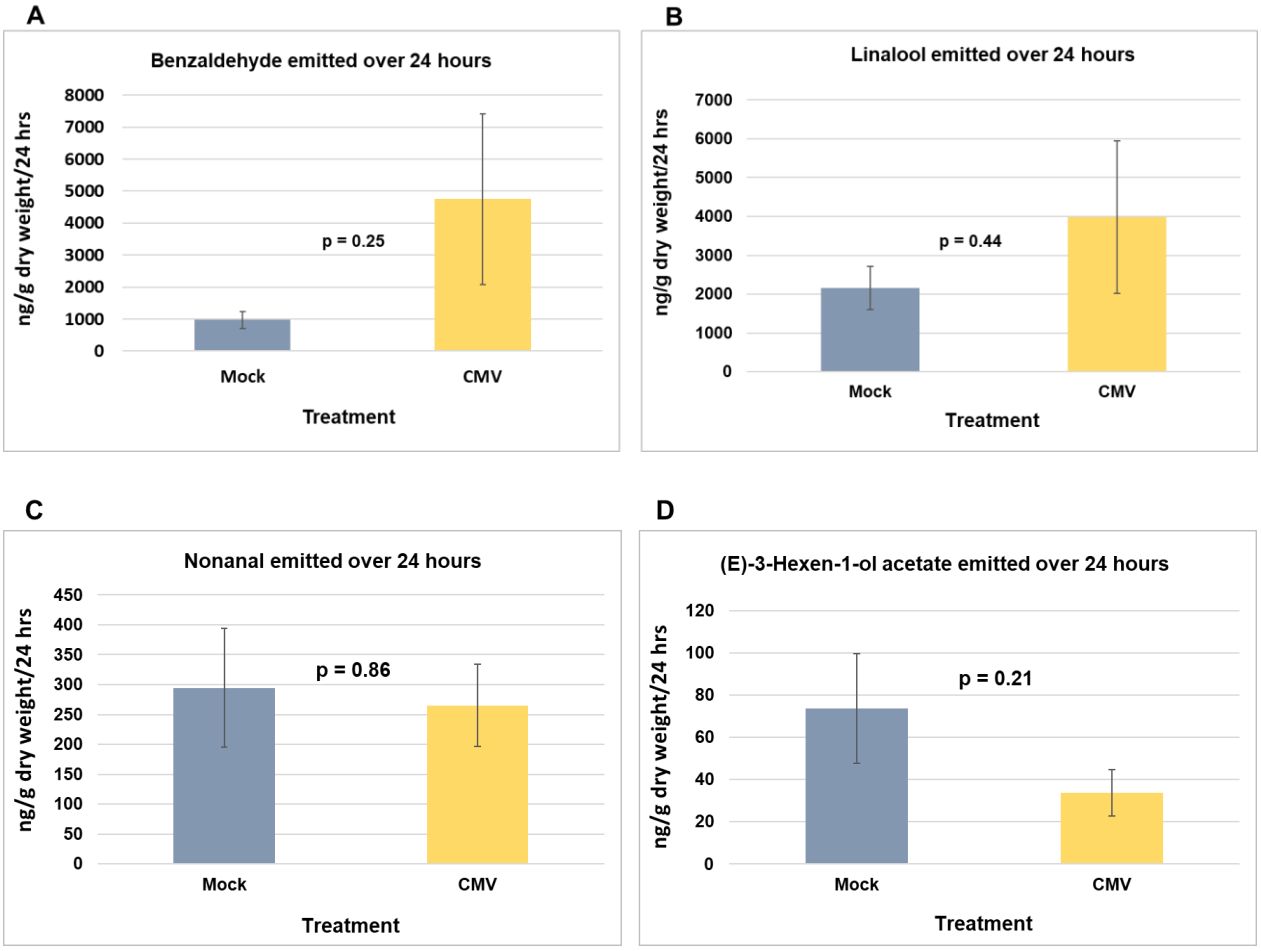


Figure 4.4 The relative production of electrophysiologically active VOCs in non-flowering cv. Dubbele witte. CMV infection appeared to induced elevation of benzaldehyde (A) and linalool (B) and decreased emission of nonanal (C) and 3-hexen-1-ol acetate (D) in non-flowering Dubbele witte market class A, although statistically insignificant. The p-values shown are from two-sample t-tests; (A) t = 1.41; (B) t = 0.89(C) t = 0.19; and (D) t = 1.41. Error bars represent standard error of the mean.

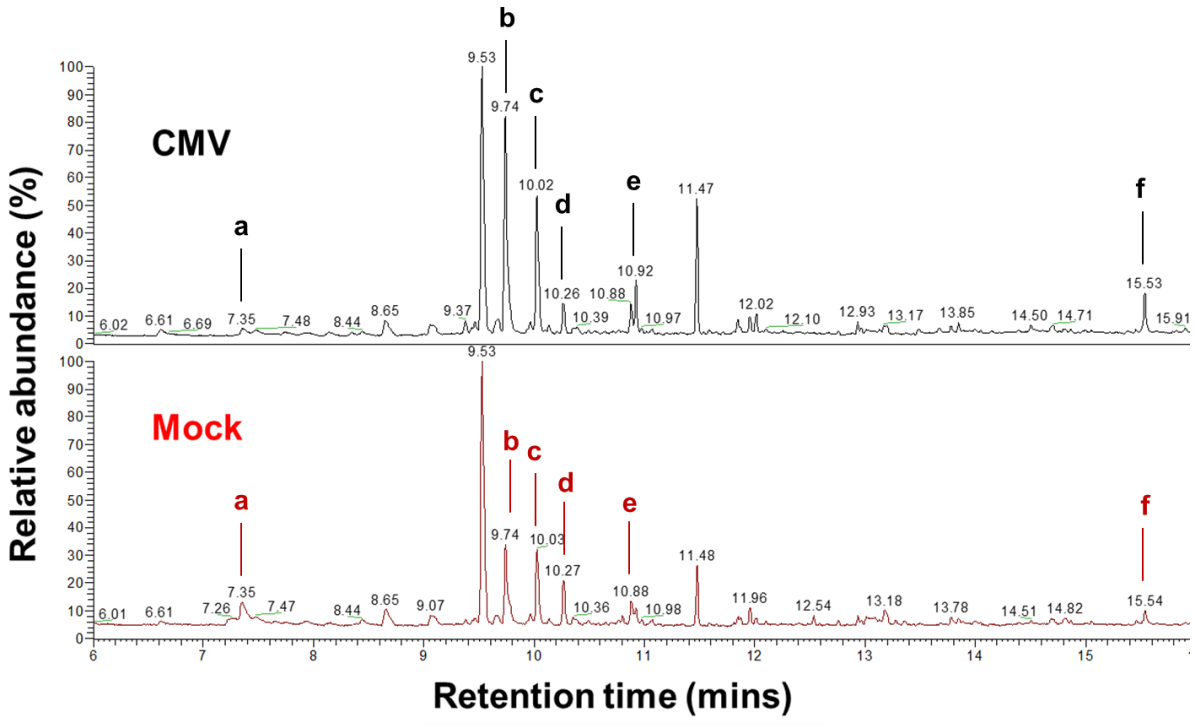


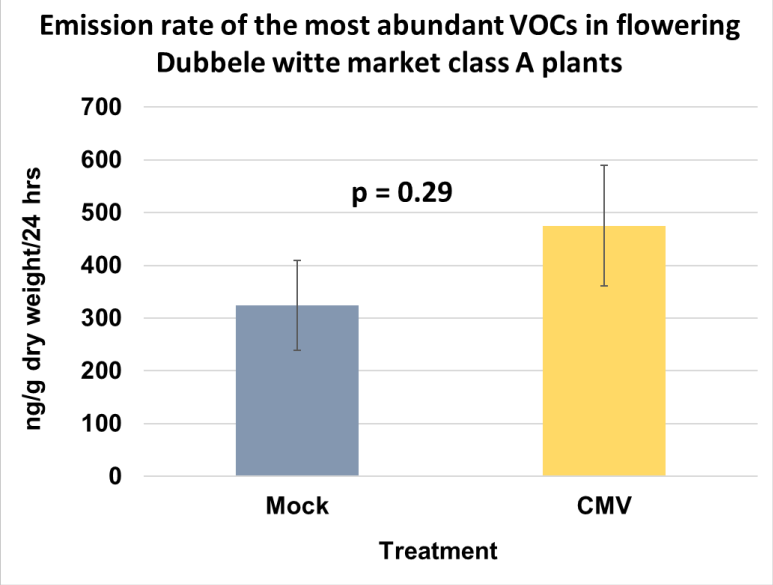
Figure 4.5 Gas chromatograms of VOCs emitted by flowering Dubbele white market class A. CMV infection appears to increase the emission of (**b**) 4-hexen-1-ol acetate (RT 9.74), (**c**) D-limonene (RT 10.02), (**e**) nonanal (RT 10.92) and (**f**) 1-isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate (RT 15.53). (**a**) (Z)-3-hexen-1-ol (RT 7.35) and (**d**) β-ocimene (RT 10.27) are slightly decreased in CMV-infected plants in comparison to mock-inoculated plants.

Table 4.2 VOCs emitted by flowering Dubbele white market class A shown in Fig 4.5

Retention time	Compound ID	Functional group
6.61	Methyl 14-(2-octylcyclopropyl)tetradecanoate	Ester
7.35	(Z)-3-Hexen-1-ol	Alcohol
8.34	sec-Butyl nitrate	Aliphatic anime
8.65	α-Pinene	Hydrocarbon
9.07	Benzaldehyde	Aldehyde
9.37	1-Octen-3-ol	Alcohol
9.53	β-Pinene	Hydrocarbon
9.74	4-Hexen-1-ol acetate	Ester
10.02	D-Limonene	Hydrocarbon
10.27	β-Ocimene	Hydrocarbon
10.88	Linalool	Alcohol
10.93	Nonanal	Aldehyde
10.97	Z,Z-3,15-Octadecadien-1-ol acetate	Ester
11.47	Siloxane	Organosilicon*
11.84	Naphthalene	Aromatic Hydrocarbon
11.96	Dodecane	Hydrocarbon
12.02	Decanal	Aldehyde
12.10	1,1-Bis(dodecyloxy)hexadecane	Ether
12.54	1,3-bis(1,1-Dimethylethyl)benzene	Aromatic Hydrocarbon
12.93	Unknown	Unknown
13.18	Unknown	Unknown

13.78	Unknown	Unknown
13.85	Tetradecane	Hydrocarbon
15.53	1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate	Ester

*Likely contaminant from equipment

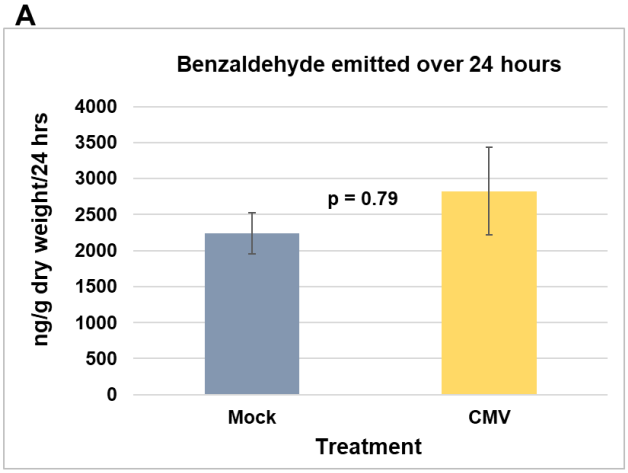


List of the most abundant VOCS

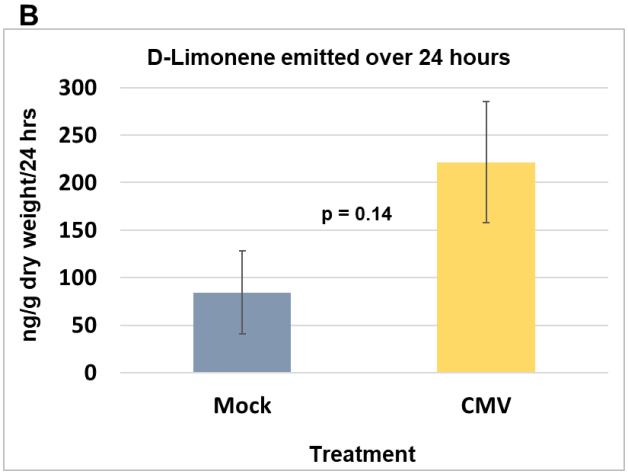
Methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.61)
(Z)-3-Hexen-1-ol (RT 7.35)
sec-Butyl nitrate (RT 8.34)
α-Pinene (RT 8.66)
Benzaldehyde (RT 9.07)
β-Pinene (RT 9.53)
4-Hexen-1-ol acetate (RT 9.74)
D-Limonene (RT 10.02)
β-Ocimene (RT 10.27)
Linalool (RT 10.88)
Dodecane (RT 11.96)
Decanal (RT 12.02)
Unknown VOC (RT 13.17)

Figure 4.6 Emission rate over 24 hours of the most abundant VOCs in flowering Dubbele witte market class A. The rate at which the most abundant VOCs was emitted in CMV-infected plants in 24 hours was not significantly different from that of mock-inoculated plants. Two-sample t-test was used to compare the emission rates of these two treatments, and the p-value is shown (t = 1.06; df = 102). Error bars represent standard errors of means.

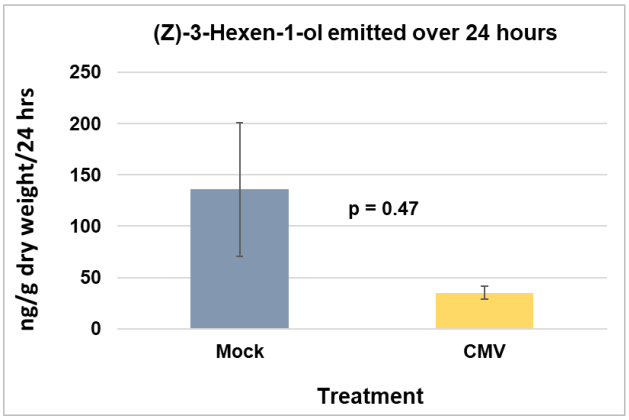
A


Benzaldehyde emitted over 24 hours
ng/g dry weight/24 hrs
4000
3500
3000
2500
2000
1500
1000
500
0
p = 0.79
Mock
CMV
Treatment

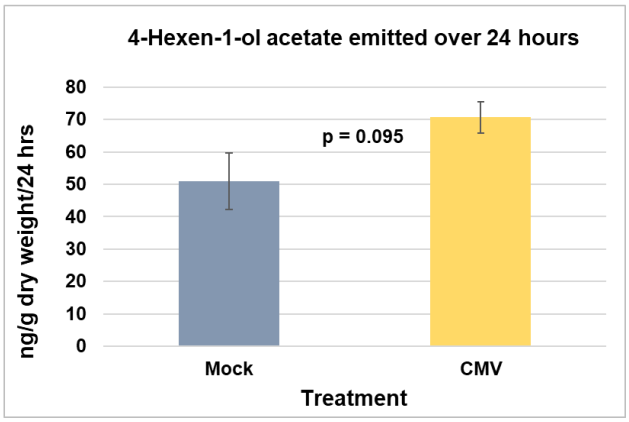
B


D-Limonene emitted over 24 hours
ng/g dry weight/24 hrs
300
250
200
150
100
50
0
p = 0.14
Mock
CMV
Treatment

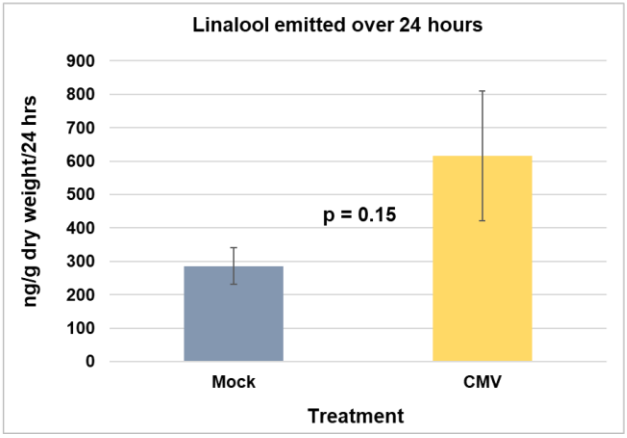
C


(Z)-3-Hexen-1-ol emitted over 24 hours
ng/g dry weight/24 hrs
250
200
150
100
50
0
p = 0.47
Mock
CMV
Treatment

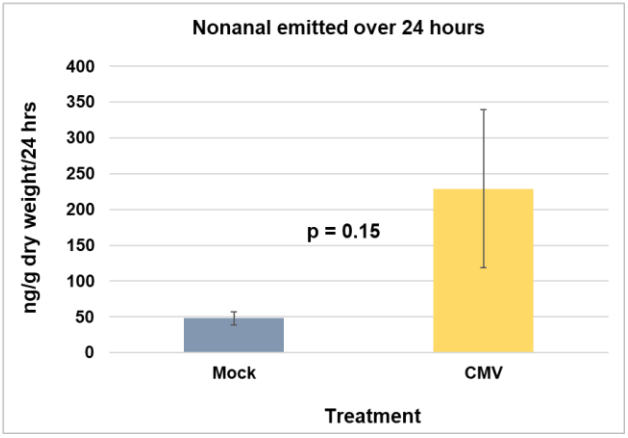
D


4-Hexen-1-ol acetate emitted over 24 hours
ng/g dry weight/24 hrs
80
70
60
50
40
30
20
10
0
p = 0.095
Mock
CMV
Treatment

E


Linalool emitted over 24 hours
ng/g dry weight/24 hrs
900
800
700
600
500
400
300
200
100
0
p = 0.15
Mock
CMV
Treatment

F


Nonanal emitted over 24 hours
ng/g dry weight/24 hrs
400
350
300
250
200
150
100
50
0
p = 0.15
Mock
CMV
Treatment

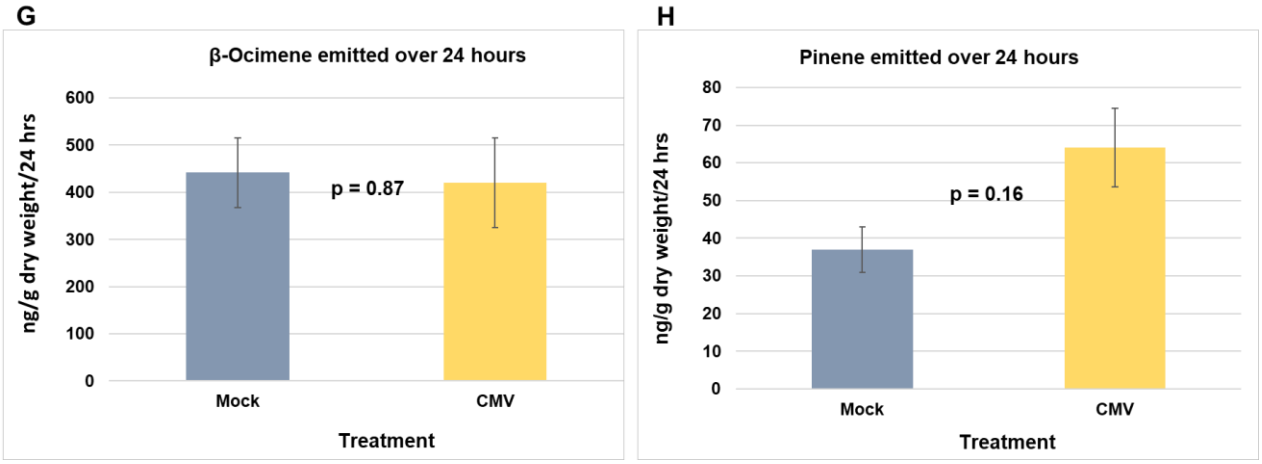


Figure 4.7 CMV-induced quantitative changes in the emission of electrophysiologically active VOCs in flowering Dubbele witte market class A plants. VOC emission rate (ng/24 hrs) per gram dry weight of eight electrophysiologically active volatiles from mock-inoculated and CMV-infected Dubbele witte market class A plants. (A) Benzaldehyde, (B) D-limonene, (D) 4-hexen-1-ol acetate, (E) linalool, (F) nonanal, and (H) pinene emissions were elevated in CMV-infected plants, but not statistically significant. (C) (Z)-3-hexen-1-ol and (G) β-ocimene emissions are reduced in CMV-infected plants but not to significant levels in comparison to mock-inoculated plants. The mean VOC emission values for individual volatiles are presented (n = 3 plants per treatment). Error bars represent standard error of the mean. The level of significance is shown by a p-value calculated with two-sample t-tests.

VOCs emitted by non-flowering Dubbele witte market class B were altered by virus infection. PCA showed that the VOC blend from BCMV-infected plants is distinct from those of mock-inoculated plants and of BCMNV-inoculated plants (Figure 4.8A). There were overlaps of ellipses of BCMNV-infected and mock-inoculated plants, but samples from each treatment group clustered together and separately from the other treatment group. The samples from each treatment group did not occupy the same positions in the scatter plot (Figure 4.8A). The PCA for VOCs from flowering Dubbele witte market class B showed low variation of 40.9% (PC 1 + PC 2). Although ellipses of BCMV and BCMNV-infected plant samples overlapped with those of mock-inoculated plants, the VOC profiles were distinct and did not occupy the same position in the scatter plot (Figure 4.8B).

In non-flowering plants, the most abundant groups of VOCs that were detected by GC-MS were esters, aromatic hydrocarbons, ketones and aldehydes (Table 4.3). Only two phenols, one alcohol, aliphatic amine and ether were detected. Twenty-four VOCs were detected, twenty- three of them were identified, one being a common contaminant, siloxane (RT 11.44) (Figure 4.9 & Table 4.3). VOCs that appeared to be commonly emitted in larger quantities in BCMV and BCMNV-infected plants in comparison to those from mock-inoculated plants, although not statistically tested, are methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.55), sec-butyl nitrate(RT 8.30); 2-ethyl-1-hexanol (RT 10.01) and 2-methyl-propanoic acid, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester (RT 15.52) (Figure 4.9 & Table 4.3). In BCMNV-infected plants alone, some VOCs appeared to be emitted in larger quantities in comparison to mock-inoculated and BCMV-infected plants, although this was not statistically proven. These are nonanal (RT 10.88), 4-ethyl-benzaldehyde (RT 11.55), benzenepropanal (RT 11.72), naphthalene (RT 11.79), oxirane (RT 11.91); 2-methyl-6-(2-propenyl)-phenol (RT 12.58), 1-(4-ethylphenyl)ethanone (RT 12.77), 1-methyl naphthalene (Rt 12.91), and 1,1'-(1,3-phenylene)bis-ethanone (RT 14.16) (Figure 4.9 & Table 4.3). The emission rates of the most abundant VOCs and those that are known to be electrophysiologically active were calculated over 24 hours

(Section 4.0 & Figure 4.10). BCMV-infected plants emitted significantly larger quantities of the most abundant VOCs in comparison to mock-inoculated plants (Figure 4.10 A). BCMV infection induced a marked increase in the emission of the most abundant VOCs (Figure 4.10 A). Virus infection did not significantly increase the emission of 2-ethyl-1-hexanol and nonanal (Figures 4.10 B).

In flowering plants, nineteen VOCs were detected by GC-MS and eighteen of them were identified, with one being a common contaminant, siloxane (RTs 11.45 & 13.17) (Figure 4.11 & Table 4.4). One VOC could not be identified using the NIST library database search. Esters, hydrocarbons and alcohols were the most abundant functional groups of VOCs that were identified in flowering plants (Figure 4.11 & Table 4.4). Other groups were only identified once, and these are; aldehyde, steroid, ketone and phenol (Figure 4.11 & Table 4.4). There were notable differences in which BCMV and BCMNV infection altered quantitative emission of VOCs in Dubbele witte market class B when compared to mock-inoculated plants (Figure 4.10). BCMNV infected induced the emission of several VOCs in larger quantities than BCMV infection. VOCs that seemed to be emitted in larger quantities as represented by their relative abundances in BCMNV-infected plants when compared to those from mock-inoculated plants are methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.56), (Z)-3-hexen-1-ol (RT 7.31), sec-butyl nitrate (RT 8.31); (Z)-3-hexen-1-ol acetate (RT 9.70), and 1-Isobutyl 4-isopropyl 3-isopropyl-2,2- dimethylsuccinate (RT 15.51) (Figure 4.10 & Table 4.4). β-Pinene (RT 9.49) was emitted in smaller quantities in BCMNV-infected samples when compared to mock-inoculated samples (Figure 4.10 & Table 4.4). BCMV-infected plants emitted β-pinene (RT 9.50) in larger quantities and (Z)-3-hexen-1-ol acetate (RT 9.71) in smaller quantities in comparison to mock-inoculated plants (Figure 4.11 & Table 4.4).

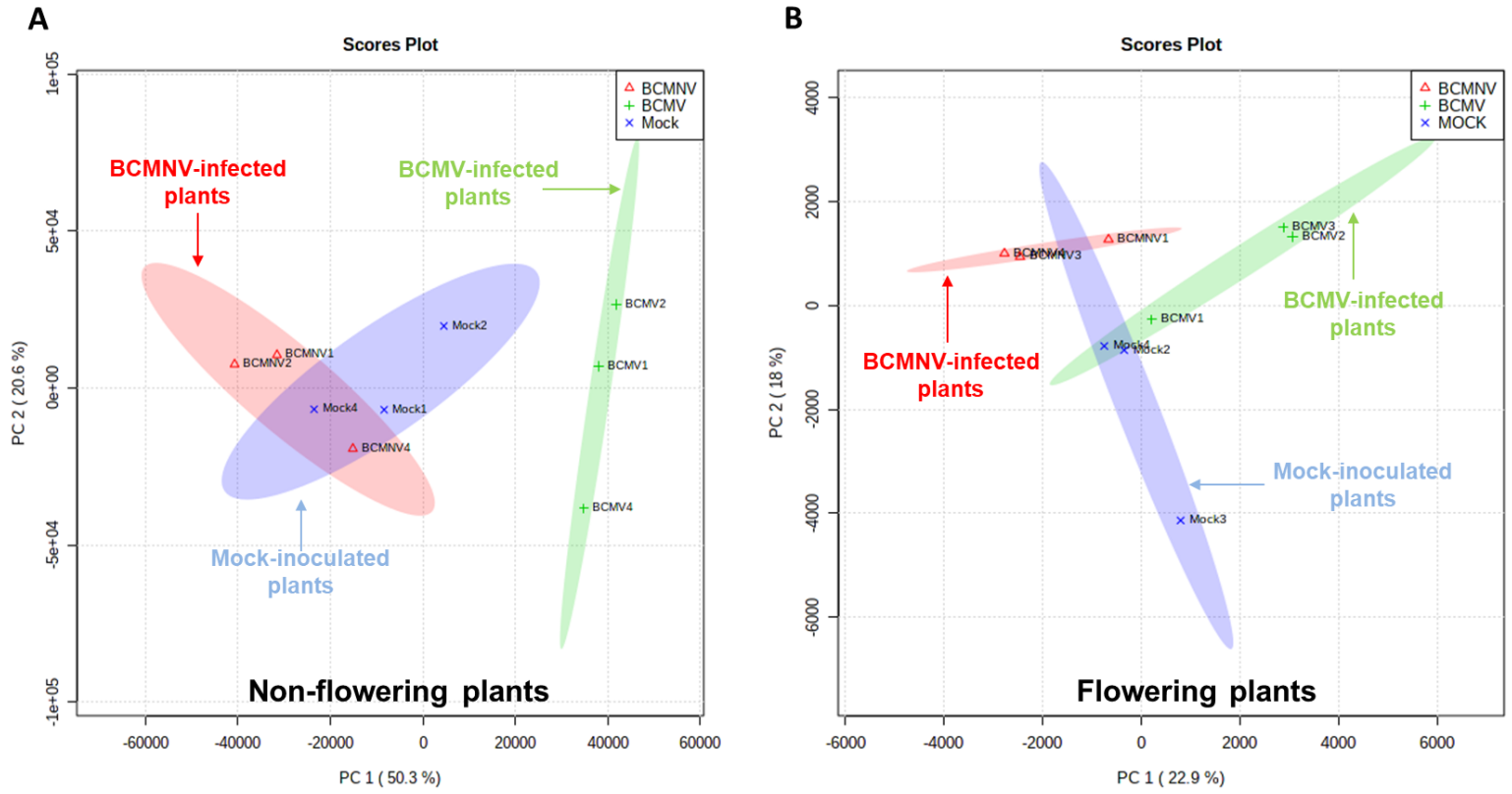


Figure 4.8 Principal component analysis of mass spectrometry data shows qualitative differences in VOC blends emitted by mock-inoculated and BCMV and BCMNV-infected Dubbele witte bean market class B in (A) non-flowering and (B) flowering plants. The cluster ellipses were generated at 95% confidence intervals and show samples from the same treatment grouping together. (A) In non-flowering plants, the volatile blend from mock-inoculated plants (light blue colour) is distinct from those of BCMV-infected

plants (light green colour). Ellipses of VOCs from mock-inoculated plants and BCMNV-infected plants (red colour) overlap, but individual samples from the two different treatment group occupy distinct spaces in the scatter plot. The principal ions representing the volatile blends from the virus-infected plants are distinct from each other. The two principal components were enough to explain 70.7% of total variability from the VOCs dataset, 50.3% by PC1 and 20.6% by PC2. (B) When plants are in flower, principal ions of VOCs blend from mock-inoculated plants (light blue) are distinct from those of BCMV-infected plants (light green colour) and BCMNV-infected plants (red colour), although the ellipses overlap. PC1 and PC2 explain only 40.9% of the total variation of the treatments.

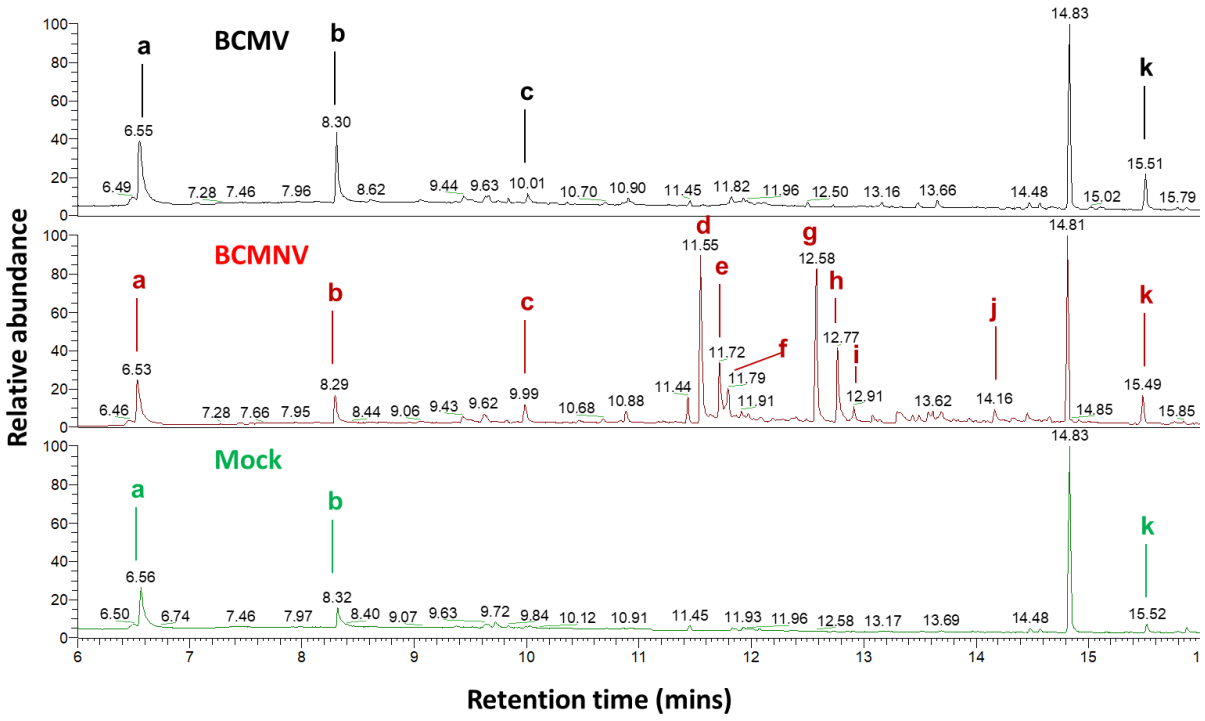


Figure 4.9 Gas chromatograms of VOCs emitted by non-flowering Dubbele witte market class B. BCMNV-infected plants (red) emit some VOCs that are not present in mock-inoculated plants (green) and BCMV-infected plants (black). These are (**d**) 4-ethylbenzaldehyde, (**e**) benzenepropanal, (**f**) naphthalene, (**g**) 2-methyl-6-(2-propenyl)phenol, (**h**) 1-(4-ethylphenyl)ethenone, (**i**) oxirane, and (**j**) m-acetyl acetophenone. VOCs from mock-inoculated plants and BCMV-infected plants are similar but vary in their relative abundances. (**a**) Methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.53 - 6.55), (**b**) sec-butyl acetate (RT 8.29 - 8.32) and (**k**) 1-isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate (RT 15.49 - 15.52) appear to be more abundant in BCMV and BCMNV-infected plants in comparison to mock-inoculated plants. (**c**) 2-ethyl-1-hexanol (RT 9.99 - 10.02) is only emitted by BCMV and BCMNV-infected plants.

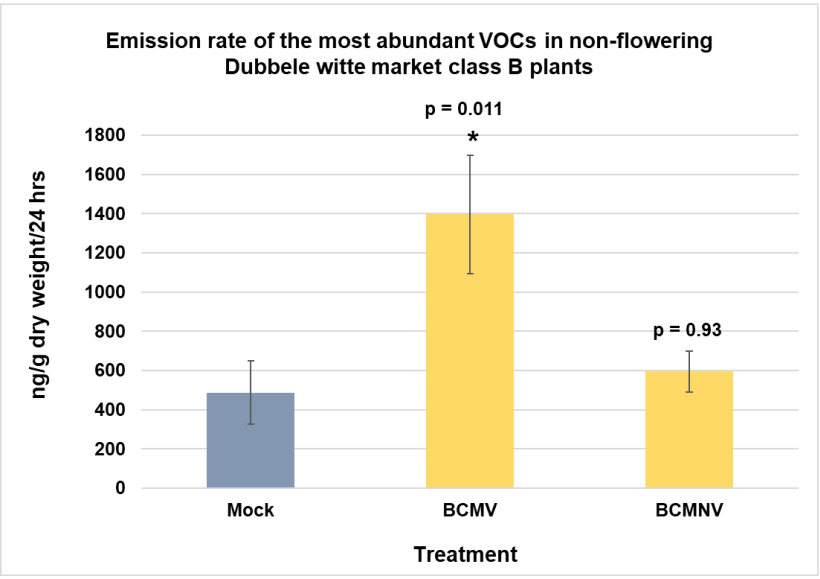
Table 4.3 VOCs emitted by non-flowering Dubbele witte market class B for Figure 4.9.

Retention time (mins)	Predicted compound	Compound type
6.56	Methyl 14-(2-octylcyclopropyl)tetradecanoate	Ester
8.32	sec-Butyl acetate	Aliphatic anime
9.43	Phenol	Phenol
9.99	2-ethyl-1-hexanol	Alcohol
10.68	4-ethenyl-1,2-dimethylbenzene	Aromatic hydrocarbon
10.88	Nonanal	Aldehyde
11.44	Siloxane	Organosilicon*
11.55	4-ethylbenzaldehyde	Aldehyde
11.72	Benzenepropanal	Aldehyde
11.79	Naphthalene	Aromatic hydrocarbon
11.91	Oxirane	Ether
12.5	1,3-bis(1,1-Dimethylethyl)benzene	Aromatic hydrocarbon
12.58	2-Methyl-6-(2-propenyl)phenol	Ketone
12.77	1-(4-ethylphenyl)ethenone	Ketone
12.91	1-Methylnaphthalene	Aromatic hydrocarbon
13.16	2-Myristynoyl pantetheine	
13.30	1-(3,4-dimethylphenyl)ethanone,	Ketone
13.69	2-Methylpropanoic acid, 3-hydroxy-2,4,4-trimethylpentyl ester	Ester
14.16	m-Acetyl acetophenone	Ketone
14.48	Unknown	Unknown
14.83	Butylated hydroxytoluene	Phenol
15.52	1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate	Ester

15.85	10,13-Octadecadienoic acid methyl ester	Ester

*Likely contaminant from equipment.

A



List of the most abundant VOCS

Methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.55)

sec-Butyl acetate(RT 8.32)

2-ethyl-1-hexanol (RT 9.99)

Nonanal (RT 10.88)

Un-identified VOC (RT 14.48)

10,13-Octadecadienoic acid methyl ester (RT 15.51)

B

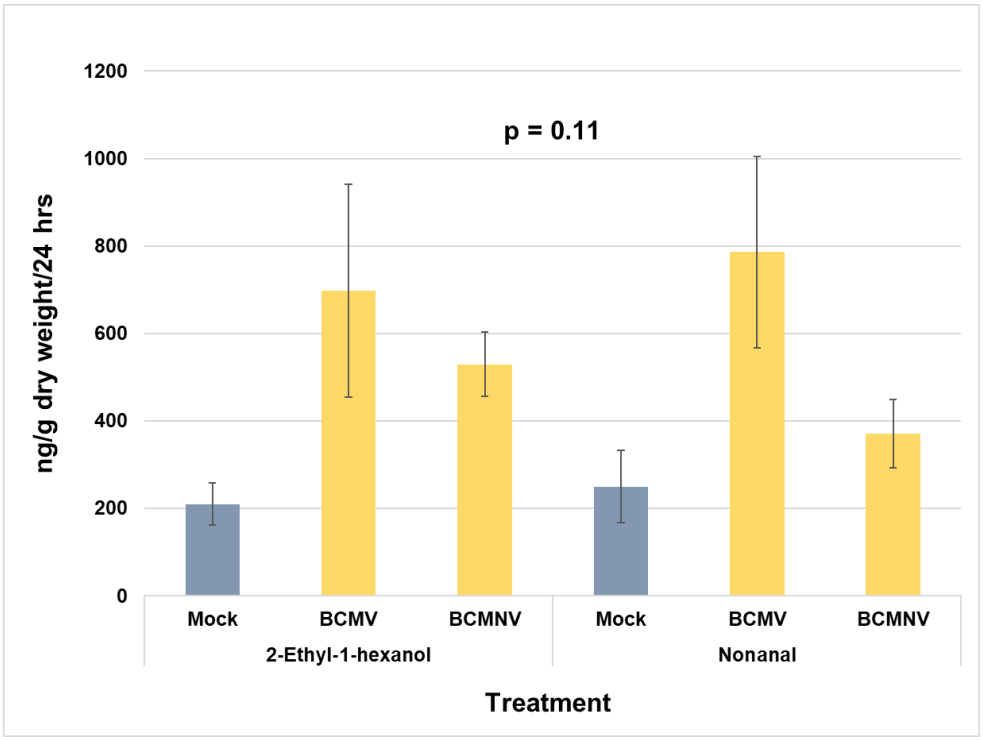


Figure 4.10 Emission rates of the most abundant and electrophysiologically active VOCs per gram dry weight of leaves in non-flowering Dubbele witte market class B. (A) BCMV infection significantly increased the emission of the most abundant VOCs as confirmed by one-way ANOVA [F (2, 21) = 5.8036, p = 0.0047). The mean VOC emission values for combined or individual volatiles are presented (n = 4 plants per treatment). The level of significance is shown

by a p-value calculated with one-way ANOVA and post-hoc Tukey's HSD testing. (B) MANOVA testing showed no significant difference in the emission of 2-ethyl-1-hexanol and nonanal among the treatment groups [F (2, 21) = 0.137]. Error bars represent standard error of the mean.

Several VOCs emissions that appeared to be decreased in virus-infected plants in comparison to those from mock-inoculated plants are linalool (RT 10.85); dodecane (RT 11.93); p-pentylacetophenone (RT 12.50); pentadecane (RT 12.73); 2,6,10-trimethyltetradecane (RT 3.24); eicosane (RT 14.66); and 1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate (RT 15.51) (Figure 4.11 & Table 4.4).

The most abundant VOCs, including known electrophysiologically active ones, were quantified and expressed as emission rate in 24 hours (Section 4.0; Figures 4.12). Overall emission of the most abundant VOCs was increased in virus-infected plants. (Z)-3-hexen-1-ol acetate and VOCs that are known to attract bees (linalool, β-ocimene and β-pinene) (see Section 4.4) were decreased in virus-infected plants (Figure 4.12 B - E). Notably, linalool was significantly decreased in BCMV-infected plants (Figure 4.12 B), and β-pinene was significantly decreased in BCMNV-infected plants (Figure 4.12 E).

In Wairimu, VOC blends from flowering CMV and BCMV-infected plants clustered distinctly from those of mock-inoculated plants (Figure 4.13). There seemed to be some similarities in the VOCs emitted by BCMNV-infected plants and mock-inoculated plants as evidenced by the overlapping of their ellipses drawn at 95% confidence intervals (Figure 4.13). There were variations on quantities of VOCs emitted by virus-infected plants when compared to those from mock-inoculated plants. VOCs that appeared to be increased in all virus-infected plants were methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.61); sec-butyl nitrate (RT 8.33); and benzaldehyde.

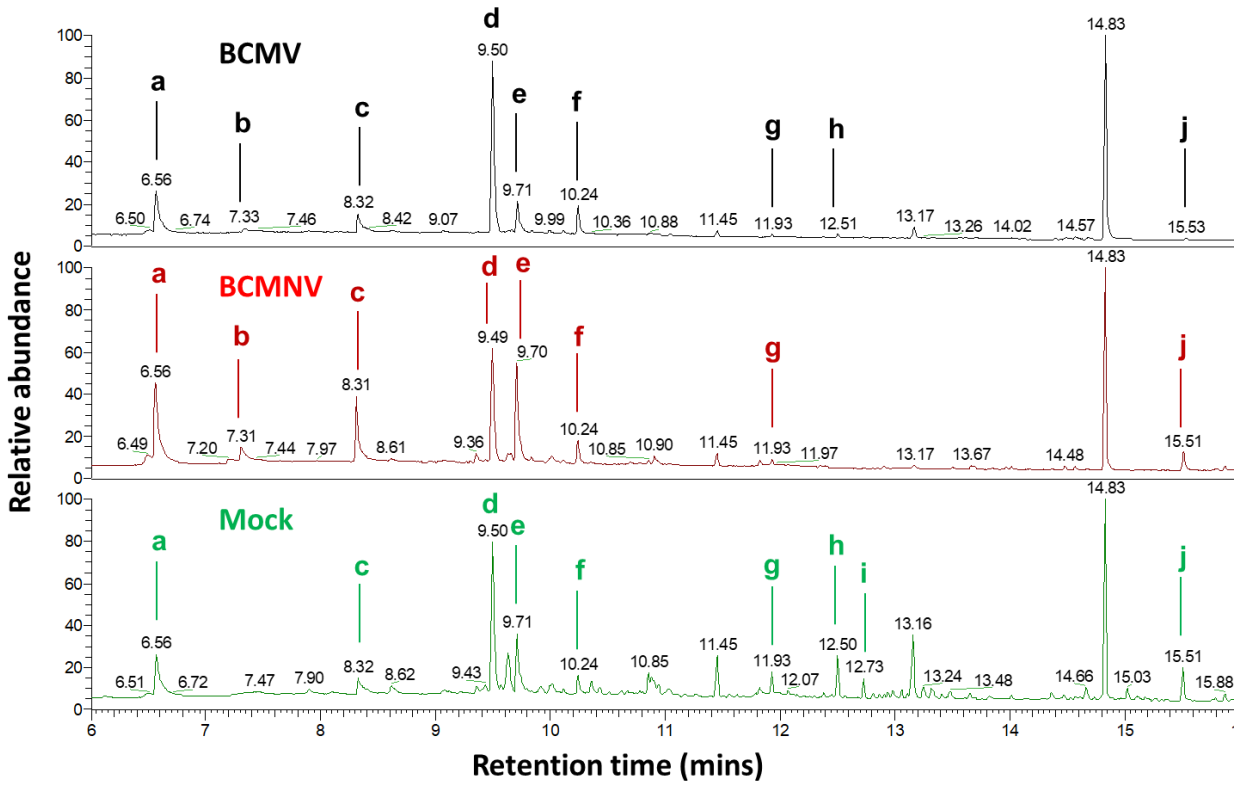


Figure 4.11 Gas chromatograms of VOCs emitted by flowering Dubbele witte market class B. Virus-induced notable changes in relative abundances of individual VOCs in virus-infected plants. In BCMNV-infected plants, the emission of (**a**) methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.56); (**b**) (Z)-3-hexen-1-ol (RT 7.31); (**c**) sec-butyl nitrate (RT 8.31), and (**e**) (Z)-3-hexen-1-ol acetate (RT 9.70) was increased in comparison their emissions in mock-inoculated plants. BCMV and BCMNV infection induced a reduction in the emission of (**g**) dodecane (RT 11.93), (**h**) p-pentylacetophenone (RT 12.50), (**i**) pentadecane (RT 12.73), and (**j**) 1-isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate (RT 15.51). When compared to emissions in mock-inoculated plants, (**d**) β-pinene (RT 9.49) seemed to be reduced in BCMNV-infected plants whereas (**f**) β-ocimene (RT 10.24) appeared to be increased in BCMV-infected plants.

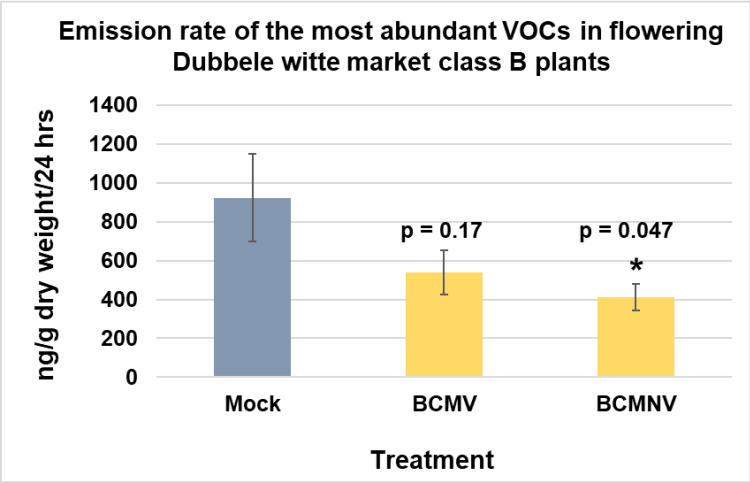
Table 4.4 VOCs list for flowering DWB market class B from Figure 4.11.

Retention time	Compound ID	Functional group
6.56	Methyl 14-(2-octylcyclopropyl)tetradecanoate	Ester
7.20	Sarreroside	Ester
7.31	(Z)-3-Hexen-1-ol	Alcohol
8.31	sec-Butyl nitrate	Aliphatic anime
9.05	Benzaldehyde	Aldehyde
9.37	1-Octen-3-ol	Alcohol
9.50	β-Pinene	Hydrocarbon
9.62	Decane	Hydrocarbon
9.71	(Z)-3-Hexen-1-ol acetate	Ester
9.99	D-Limonene	Hydrocarbon
10.13	α-Pinene	Hydrocarbon
10.24	β-Ocimene	Hydrocarbon
10.38	Ethyl iso-allocholate	Steroid
10.88	Linalool	Alcohol
11.45	Siloxane	Organosilicon*
11.93	Dodecane	Hydrocarbon
12.50	p-Pentylacetophenone	Ketone
12.73	Pentadecane	Hydrocarbon
13.17	Siloxane	Organosilicon*

13.24	2,6,10-trimethyltetradecane	Hydrocarbon
14.60	2,2,7,7-Tetramethyltricyclo[6.2.1.0(1,6)]undec-4-en-3-one	Ketone
14.66	Eicosane	Hydrocarbon
14.87	Butylated Hydroxytoluene	Phenol
15.51	1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate	Ester
15.53	Unknown	Unknown
15.88	Methyl 8,11,14,17-eicosatetraenoate	Ester

*Likely contaminant from equipment

A



List of the most abundant VOCS
Methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.56)
β-Pinene (RT 9.50)
Isopropyl acetate (RT 8.32)
(Z)-3-Hexen-1-ol acetate (RT 9.71)
D-Limonene (RT 9.99)
β-Ocimene (RT 10.24)
Linalool (RT 10.88)
Dodecane (RT 11.93)
p-Pentylacetophenone (RT12.50)
Methyl 8,11,14,17-eicosatetraenoate (RT 15.51)

B

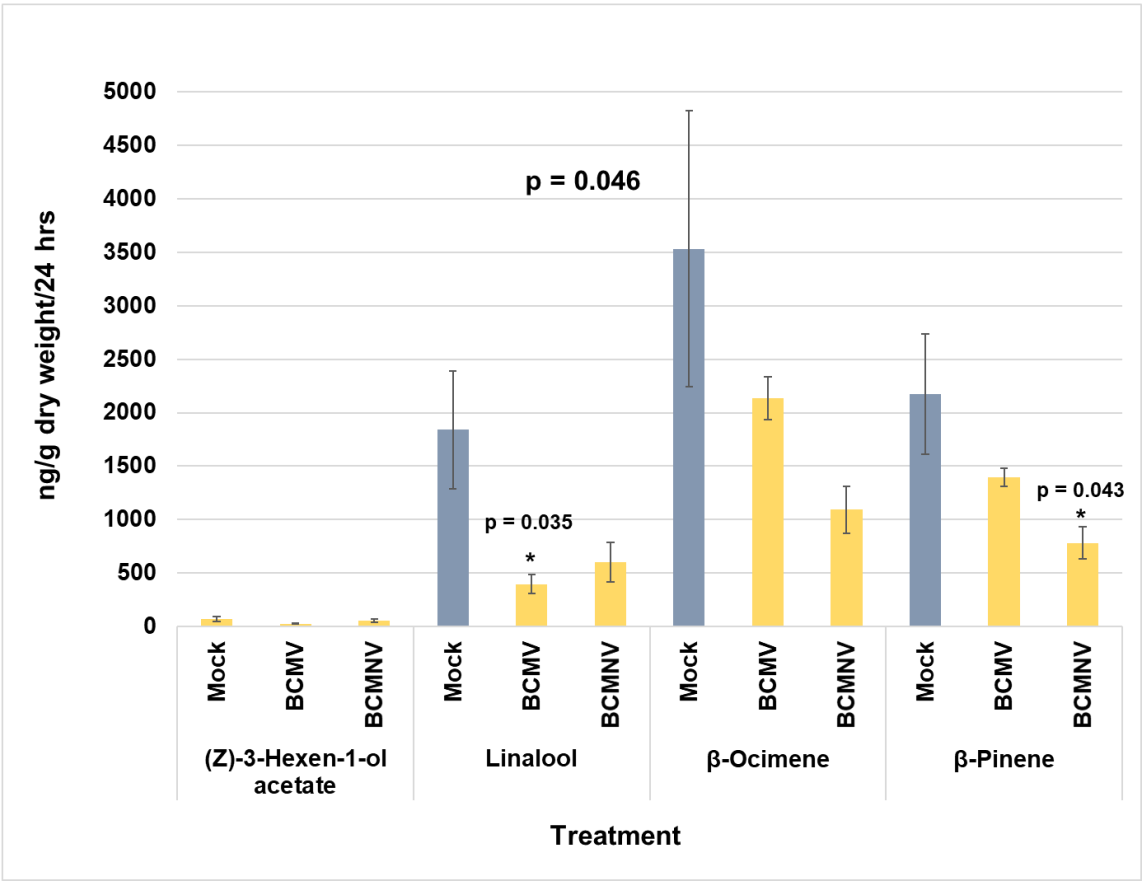


Figure 4.12 Virus-induced quantitative changes in the emission of flowering Dubbele witte market class B VOCs (A) Whole plant total emission rate (ng/24hrs) for the ten most abundant volatiles was significantly decreased in BCMNV-infected plants. (B) VOC emission rates ($ng.h^{-24}$)

per gram dry weight of the four electrophysiologically active volatiles are generally decreased in virus-infected plants and MANOVA test proved significant differences in two of the VOCs [$F(2, 7) = 4.52$, $p = 0.046$]. Notably, linalool emission was significantly reduced in BCMV-infected plants, and β-pinene emission is significantly reduced in BCMNV-infected plants. The mean VOC emission values for combined or individual volatiles are presented ($n = 3$ plants per treatment). Error bars represent standard error of the mean. The level of significance on linalool and β-pinene is shown by a p-value calculated with one-way ANOVA and post-hoc Tukey's HSD testing.

(RT 9.05) (Figure 4.14 & Table 4.5). The proportion by which methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.61) and sec-butyl nitrate (RT 8.33) were increased in BCMNV and CMV-infected plants was much higher than in BCMV-infected plants (Figure 4.14). The emission of (E)-8-dimethyl-1,3,7-nonatriene (Rt 11.07) and (E)-ocimene (RT 10.26) appeared to be decreased in all virus-infected plants when compared with mock-inoculated plants (Figure 4.14 & Table 4.5). BCMNV and CMV infection had similar effects on the quantities of some VOCs emitted by flowering Wairimu plants. Both viruses seemed to induce an increase in the emission of 3-hexen-1-ol (RT 7.35); α-pinene (RT 8.66); 4-hexen-1-ol acetate (RT 9.74); limonene (RT 10.03), and linalool (RT 10.91). Conversely, the quantities of these VOCs remained unchanged in BCMV-infected plants compared to mock-inoculated plants (Figure 4.14 & Table 4.5). Plants infected with BCMNV uniquely increased the emission of several VOCs in comparison to mock-inoculated plants. These were acetophenone (RT 10.49); dodecane (RT11.96); tetradecane (RT 12.54); unknown (RT12.76); tridecane (13.18); caryophyllene (RT13.28); 2,6,10-trimethyltetradecane (RT 13.85); unknown (RT14.50); unknown (RT14.69); ethyl iso-allocholate (RT15.06) and 1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate (RT15.54) (Figure 4.14 and Table 4.5). 4-hexen-1-ol acetate (RT 9.74) appeared to be decreased only in BCMV-infected plants.

The most abundant VOCs were quantified and expressed as emission rate over a 24 hour period in nanograms per gram dry weight of leaves and floral tissue (ng/g/24 hours) (Figure 4.15). It shows that BCMNV infection significantly increased the emission of VOC blend when compared to those of mock-inoculated plants. VOC blend emission rates from BCMV and CMV-infected plants were markedly decreased when compared to those of mock-inoculated plants (Figure 4.15). Comparisons of emission rates of VOCs that are electrophysiologically active was made and among them are VOCs known to attract bees (Section 4.0; Figure 4.16). VOCs that attract

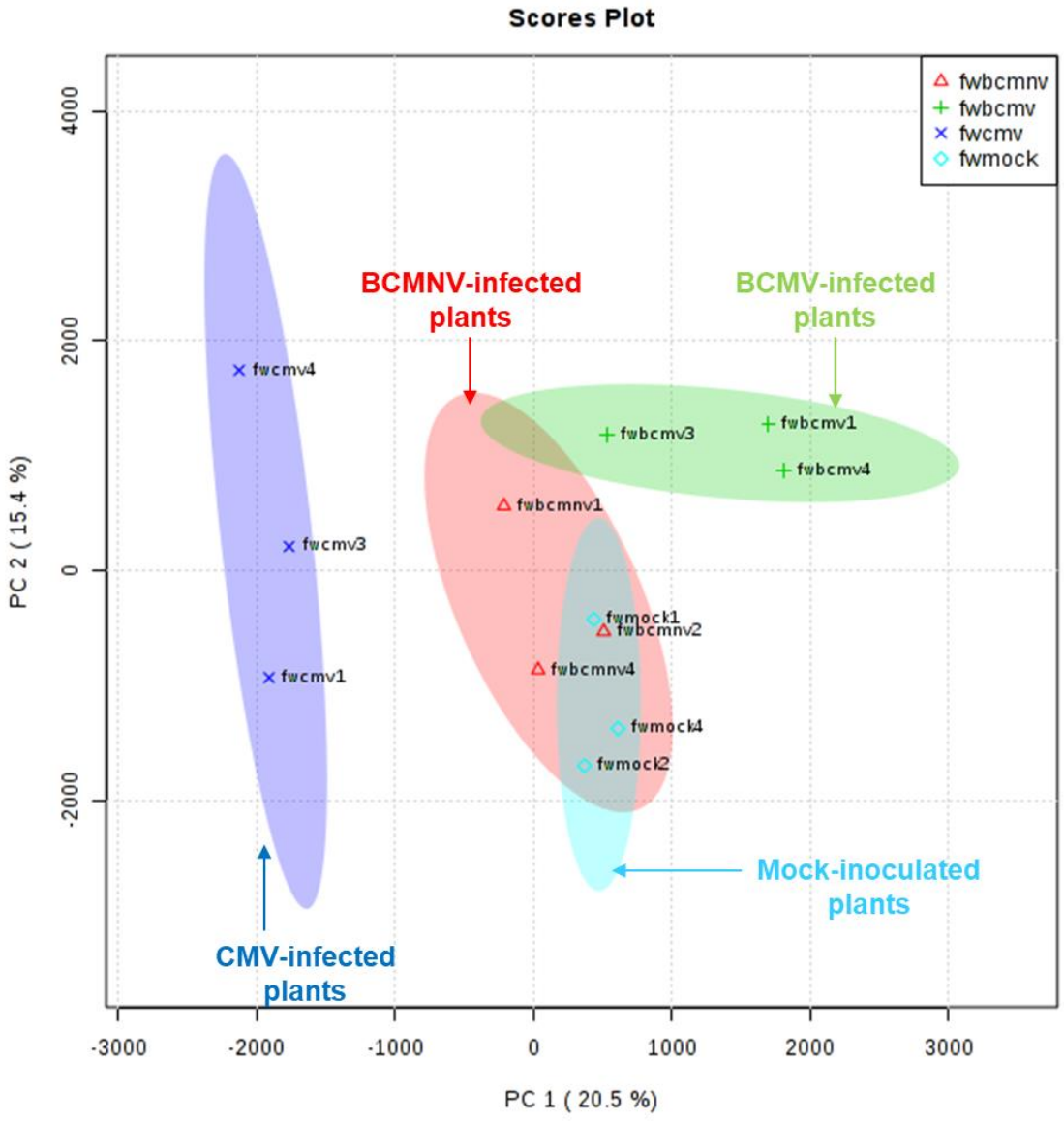


Figure 4.13 2D scatter plot from a PCA of VOCs from flowering Wairimu plants. VOC blends from CMV and BCMV-infected plants are distinct from those of mock-inoculated plants VOC blends from BCMNV-infected plants overlap with those from mock-inoculated plants. PC1 explained 20.5% and PC2 1explained 15.4% of the variation of principal VOCs among the treatments. Cumulatively, both PCs accounted for only 35.9% of the variation. The cluster ellipses were generated at 95% confidence intervals.

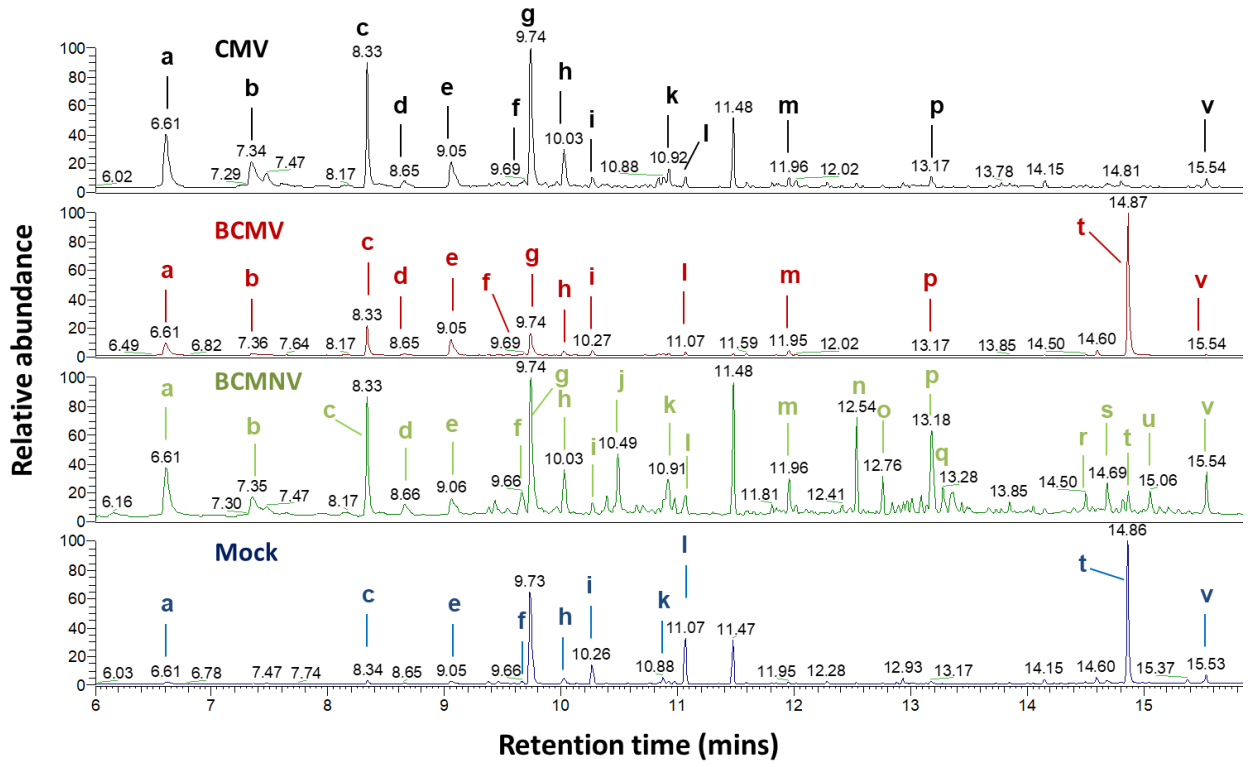


Figure 4.14 Gas chromatograms indicating relatively abundant VOCs emitted by mock-inoculated and virus-infected flowering Wairimu. Table 4.5 gives the identities of the VOCs. Notable changes where VOCs are increased in all virus-infected plants are (**a**) methyl 14-(2-octylcyclopropyl)tetradecanoate (6.61), (**c**) sec-butyl nitrate (RT 8.33), and (**e**) benzaldehyde (RT 9.05). The emission of (**l**) (E)-8-dimethyl-1,3,7-nonatriene (RT11.07) was decreased in all virus-infected plants. BCMNV-infection uniquely increased the emission of (**m**) dodecane (RT 11.96), (**n**) tetradecane (RT 12.54), (**o**) un-identified VOC (RT 12.76), (**p**) tridecane (RT13.18), (**q**) caryophyllene (RT 13.28), (**r**) un-identified VOC (RT14.50), (**s**) un-identified VOC (RT 14.69), (**u**) ethyl iso-allocholate (RT15.06), and (**v**) 4,8,12-trimethyl-1,3,7,11-tridecatetraene (RT 15.54). BCMV infection did not induce much changes, (**g**) 4-hexen-1-ol acetate (RT 9.74), (**i**) (E)-ocimene (RT 10.27), and (**r**) (E)-8-dimethyl-1,3,7-nonatriene (RT 11.07) emissions were decreased in comparison to mock-inoculated plants.

Table 4.5 Key to identities of VOCs in Figure 4.11

Retention time (mins)	Predicted compound	Compound type
6.61	Methyl 14-(2-octylcyclopropyl)tetradecanoate	Alcohol
7.26	(E)-2-Hexenal	Aldehyde
7.35	3-Hexen-1-ol	Alcohol
8.17	Methyl dodecanoate	Ester
8.33	sec-Butyl nitrate	Aliphatic anime
8.66	α-Pinene	Alkene
9.05	Benzaldehyde	Aldehyde
9.74	4-Hexen-1-ol acetate	Ester
9.96	p-Cymene	Aromatic hydrocarbon (Alkylbenzene)
10.03	Limonene	Hydrocarbon (Monoterpene)
10.26	(E)-ocimene	Hydrocarbon (Monoterpene)
10.49	Acetophenone	Ketone
10.88	Nonanal	Aldehyde
10.92	Linalool	Alcohol
11.07	(E)-8-dimethyl-1,3,7-nonatriene	Homoterpene
11.38	Benzyl acetate	Ester
11.48	Siloxane	Organosilicon*
11.96	Dodecane	Alkane hydrocarbon
12.54	Tetradecane	Alkane hydrocarbon
12.76	Unknown	Unknown
13.18	Tridecane	Alkane hydrocarbon
13.28	Caryophyllene	Sesquiterpenes
13.77	Ethyl iso-allocholate	Steroid derivative

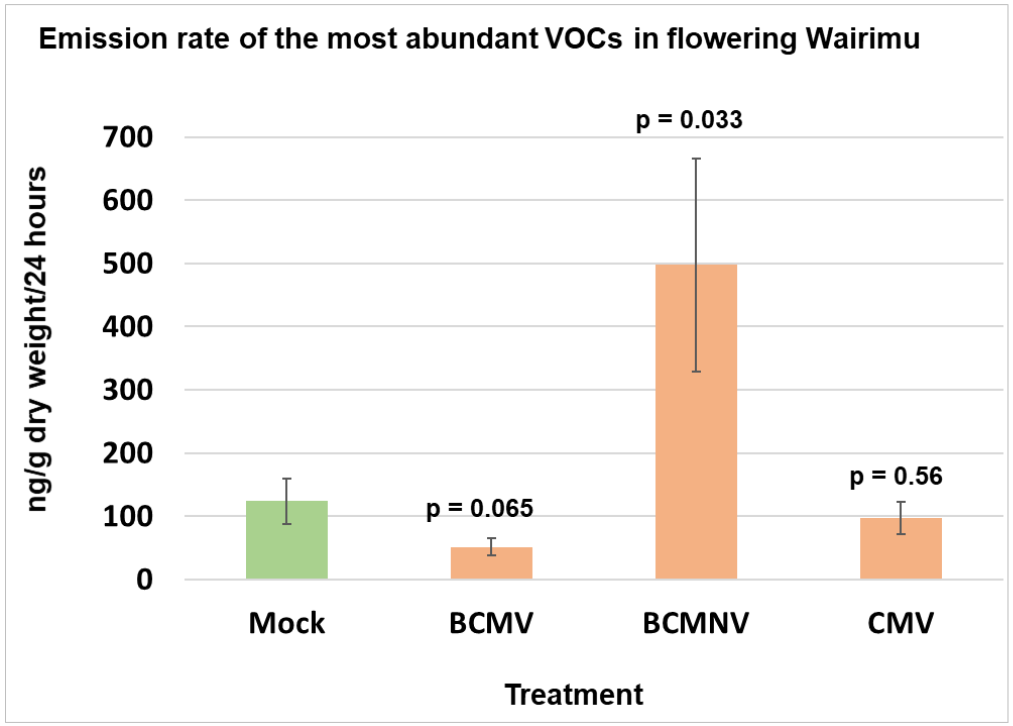
13.85	2,6,10-trimethyltetradecane	Hydrocarbon
14.50	Unknown	Unknown
14.6	2,2,7,7-Tetramethyltricyclo(6,2,1,0(1,6)undec-4-en-3-one	Ketone
14.69	Unknown	Unknown
14.87	Butylated hydroxytoluene	Phenol
15.06	Ethyl iso-allocholate	Steroid derivative
15.54	1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate	Ester
15.7	4,8,12-trimethyl-1,3,7,11-tridecatetraene	Homosesquiterpene

*Likely contaminant from equipment

bees that were identified in the blends of flowering cv. Wairimu are benzaldehyde, (E)-ocimene, D-limonene, linalool and α-pinene. All three viruses appeared to emit elevated levels of benzaldehyde (Figure 4.16) and decreased levels of (E)-ocimene (Figure 4.16). 3-Hexen-1-ol levels were significantly elevated in BCMNV and CMV-infected plants (Figure 4.16). BCMV and CMV-infected plants appeared to produced lower levels of most of the electrophysiologically active VOCs with exceptions of benzaldehyde (Figure 4.16B) and 3-hexen-1-ol acetate (Figure 4.16H), although not statistically different from those of mock-inoculated plants. Levels of D-limonene were significantly elevated in BCMNV-infected plants (Figure 4.16).

4.2.2 BCMV and BCMNV infection-induced changes in VOCs emitted by non-flowering and flowering Dubbele witte

VOC blends of non-flowering and flowering plants were compared between plants of the same treatment and bean variety. Blends from both non-flowering and flowering mock-inoculated and CMV-infected Dubbele witte market class A plants were distinct (Figure 4.17). CMV-infected plants showed greater variation as confirmed by higher PC percentages than in mock-inoculated plants. The similar trend was observed in cv. Dubbele witte market class B, with BCMNV infection inducing the greatest variation between flowering and non-flowering plants.



List of the most abundant VOCs

Methyl 14-(2-octylcyclopropyl)tetradecanoate (RT 6.61)
sec-Butyl nitrate (RT 8.33)
Benzaldehyde (RT 9.05)
(E)-Ocimene (RT 10.26)
4,8-dimethyl-1,3,7-nonatriene (RT 11.07)
3-Hexen-1-ol (RT 7.35)
α-Pinene (RT 8.66)
4-Hexen-1-ol acetate (RT 9.74)
D-Limonene (RT 10.03)
Linalool (RT 10.91)
Acetophenone (RT 10.49)
Dodecane (RT11.96)
Tetradecane (RT 12.54)
Unknown (RT 12.76)
Tridecane (RT 13.18)
Caryophyllene (RT 13.28)
2,6,10-trimethyltetradecane (RT 13.85)
Unknown (RT 14.50)
Unknown (RT 14.69)
Ethyl iso-allocholate (RT 15.06)
4,8,12-trimethyl-1,3,7,11-tridecatetraene(RT15.54)

Figure 4.15 Emission rate of the most abundant VOCs per gram dry weight of leaves and flowers in flowering Wairimu. Only BCMNV-infected plants produced larger quantities of VOC blends that were significantly different from those of mock-inoculated plants (t-test: t = 2.16; df = 118). BCMV and CMV-infected plants emitted VOV blends that were markedly decreased in comparison to those of mock-inoculated plants. BCMV vs mock treatment (t-test: t = 1.86; df = 118) and CMV vs mock treatment (t-test: t = 0.56; df = 118). The p-values shown in the graph are from two-sample t-tests of each treatment compared with mock-inoculated treatment at 95% confidence intervals. Error bars represent standard error of the mean values.

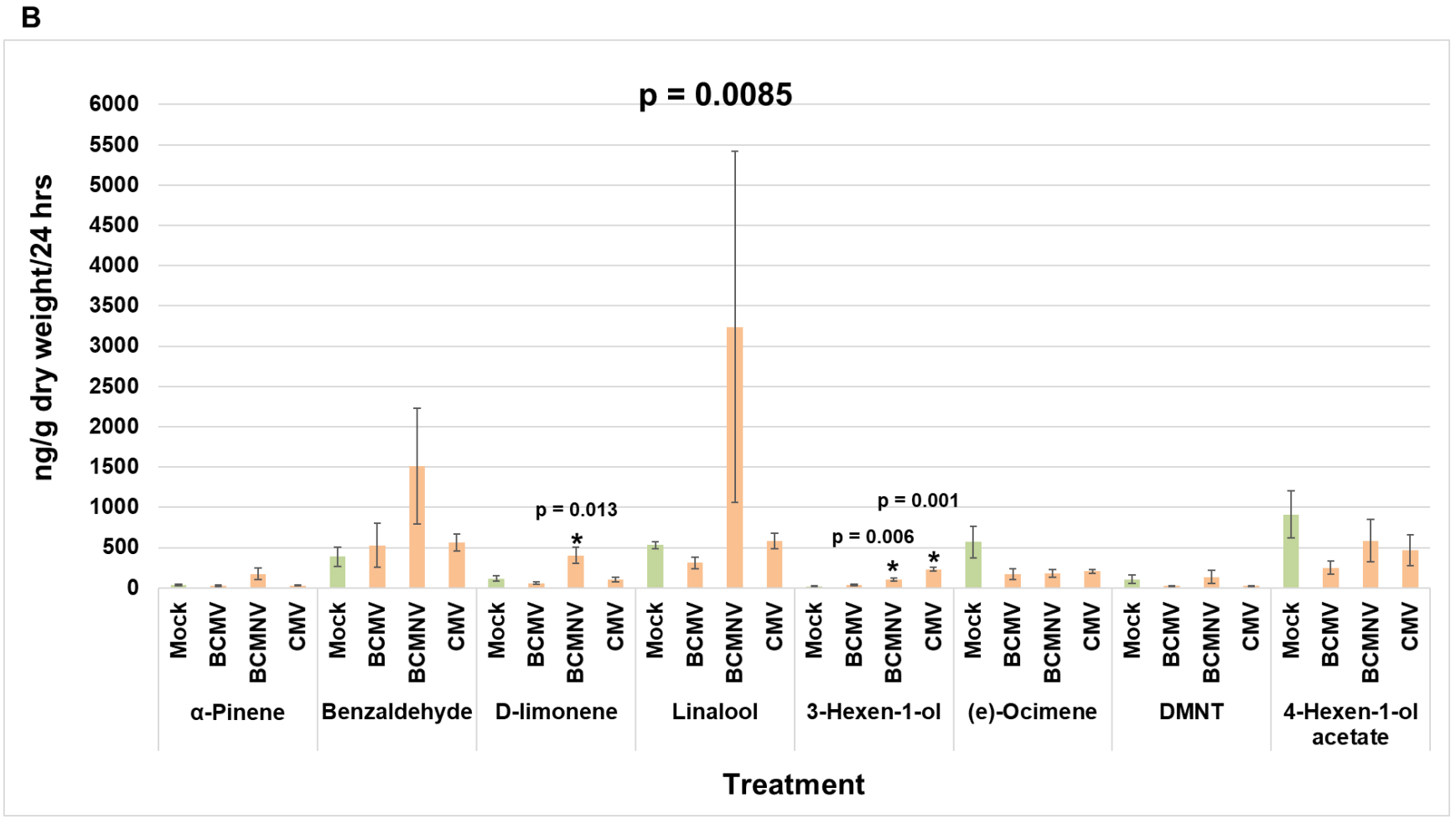


Figure 4.16 The relative production of electrophysiologically active VOCs in flowering cv. Wairimu. Among the eight volatiles presented here, three were previously identified by GC-EAG as electrophysiologically active, and five of them attract foraging bees

(Section 4.0). Virus infection engendered differences in production levels of all the eight volatiles. MANOVA testing showed that there were significant differences in the emission of D-limonene and 3-hexen-1-ol [F 0(3, 21) = 2.89]. Further one-way ANOVA and post-hoc comparisons of mock treatment versus virus treatments revealed that 3-hexen-1-ol levels were significantly increased in BCMNV and CMV-infected plants in comparison to mock-inoculated plants and D-limonene levels were significantly increased in BCMNV-infected plants. Other VOC levels varied across different virus treatments, but not statistically different from those of mock-inoculated plants. BCMNV infection appeared to elicit the most changes in levels of electrophysiologically active compounds. BCMV and CMV-infected plants emitted lower levels of six electrophysiologically active VOCs and only elevated levels of (B) benzaldehyde and (E) 3-hexen-1-ol. The relative amounts were determined by measuring the area under the curve of the peaks detected, normalising them with dry weight of leaf and floral tissue and standardised using standard curves. Quantification of benzaldehyde, linalool, α-pinene, and (E)-ocimene was done using their own purified standards, whereas the rest were quantified using Pinene-equivalent standard. The p-values shown in the graphs are from Tukey's HSD post-hoc tests of each virus treatment compared with mock-inoculated treatment at 95% confidence intervals. Error bars represent standard error of the mean values.

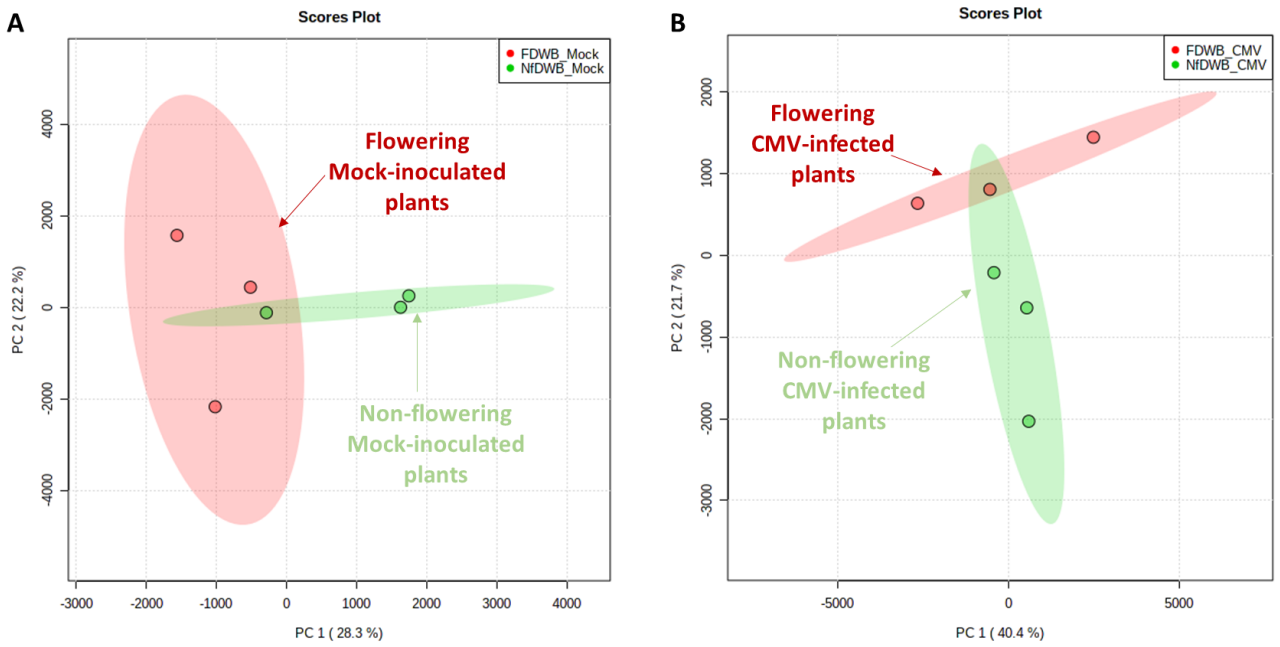


Figure 4.17 A comparison by principal component analysis of VOC blends emitted by non-flowering and flowering Dubbele witte market class A of the same treatment. (A) Mock-inoculated non-flowering and flowering plants emit overlapping VOC blends showing similarities in principal VOC components. PC1 explained 28.3% of the variation of the principal VOCs between the two treatments and PC2 22.2%. (B) Although ellipses of CMV-infected non-flowering and flowering plants overlap, the position of actual samples on the graph shows that these two treatments emit distinct principal VOCs. This variation is explained by PC1 (40.4%) and PC2 (21.7%) at a high of 62.1%. All cluster ellipses were generated at 95% confidence intervals.

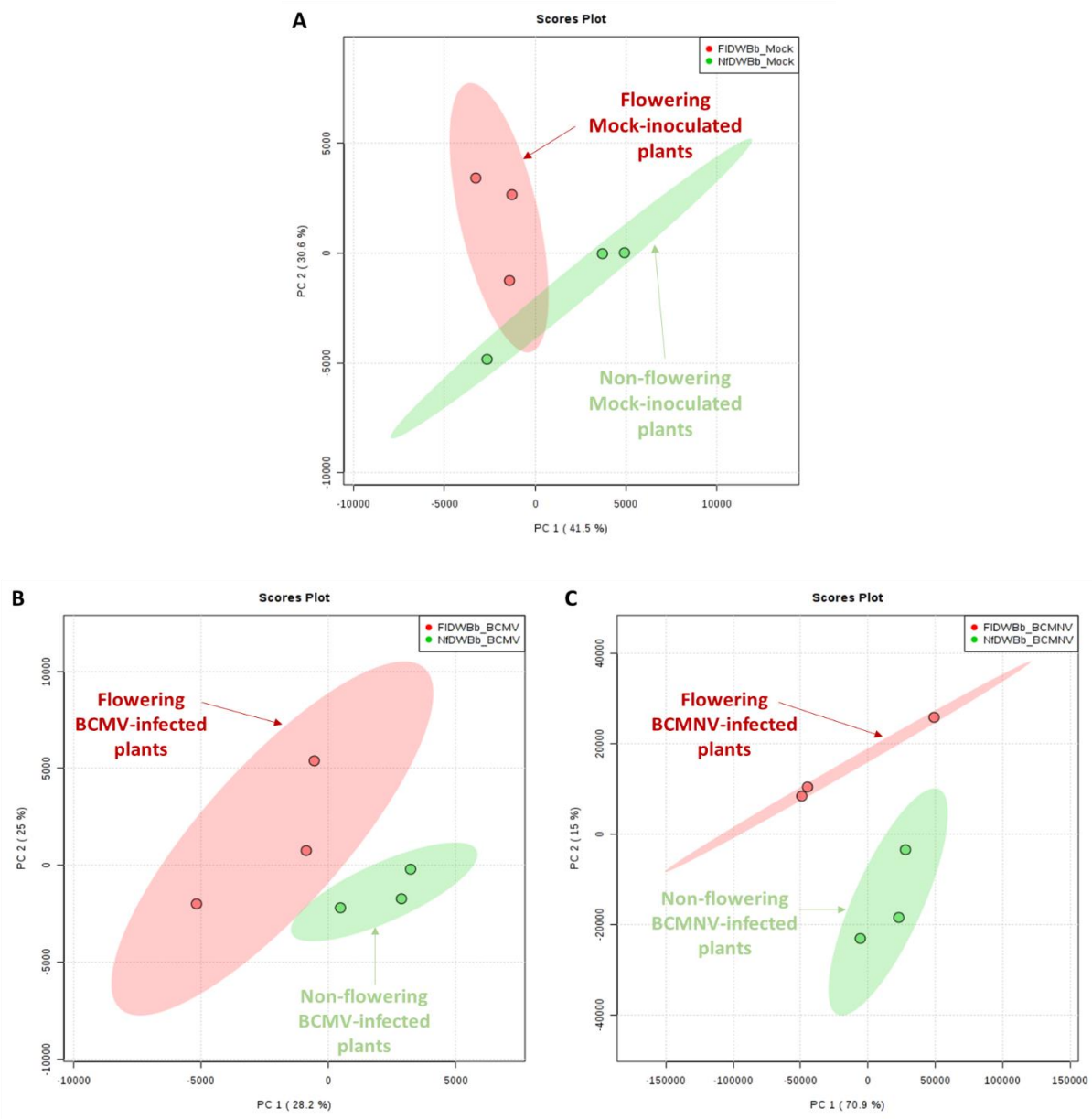


Figure 4.18 A comparison by principal component analysis of VOC blends emitted by non-flowering and flowering Dubbele witte market class B of the same treatment. (A) mock-inoculated non-flowering and flowering plants emit overlapping VOC blends showing similarities in principal VOCs they emit. (B) BCMV-infected and BCMNV-infected non-flowering and flowering plants emit VOC blends that are distinct. The samples cluster separately. (C) BCMNV-infected non-flowering and flowering plants blends differ greatly as supported by PC 1 parenthesis of 70.9 % and PC 2 15 %.

Table 4.6 Summary of results of changes in VOCs in virus-infected plants.

Treatment	BCMV	BCMNV	CMV
Dubelle witte market class A			
Non-flowering plants VOCs	N/A	N/A	Distinct from mock by 74.3% (PC 1 + PC 2). Emission rate of most abundant VOCs significantly increased.
Flowering plants VOCS	N/A	N/A	Distinct from mock by 50.8% (PC 1 + PC 2).
Dubelle witte market class B			
Non-flowering plants VOCs	Very distinct from mock VOCs. Emission rate of most abundant VOCs increased.	Distinct from mock VOCs although 95% confidence interval ellipses overlap.	N/A
Flowering plants VOCs	Distinct from mock. Emission of Linalool significantly decreased.	Distinct from mock. Emission of most abundant VOCs decreased, as well as Linalool, β-Pinene	
Wairimu			
Flowering plants VOCs	Distinct from mock. Emission rate of	Distinct from mock. Emission of most	Very distinct from mock. Emission rate

	Linalool significantly decreased.	abundant VOCs significantly increased, as well as D-Limonene and 3-Hexen-1-ol	of 3-Hexen-1-ol significantly increased.

Table 4.7 Summary of comparison of VOCs from the same treatment in non-flowering and flowering plants

Treatment	Mock	BCMV	BCMNV	CMV
Dubbele witte market class A plants				
Non-flowering vs flowering plants VOCs	Distinct with a variation of 50.5% from PC 1 + PC 2.	N/A	N/A	Distinct with a variation of 62.1% from PC 1 + PC 2.
Non-flowering DWB market class B plants				
Non-flowering vs flowering plants VOCs	Distinct with a greater variation of 72.1% from PC 1 + PC 2.	Distinct with a variation of 53.2% from PC 1 + PC 2.	Distinct with the greatest variation of 85.9% from PC 1 + PC 2.	

Bee attracting VOCs like α-Pinene, Limonene and Ocimene were only found in flowering plants, whereas Linalool and Benzaldehyde were commonly found in both flowering and non-flowering plants across all treatments (Tables 4.1 - 4.5). Typical green leaf VOCs like the hexenal family compounds were detected in both non-flowering and flowering plants of both varieties (Tables 4.1- 4.5). In both common bean varieties, methyl 14-(2-octylcyclopropyl)tetradecanoate was detected in both non-flowering and flowering plants (Tables 4.1 – 4.5).

4.3 DISCUSSION

4.3.1 Viruses induced changes in VOCs emitted by *P. vulgaris* non-flowering and flowering plants

The quality and quantity of VOC blends emitted by *P. vulgaris* were altered by BCMV, CMV and BCMNV infection (Table 4.6). CMV infection caused significant changes in the principal VOCs and quantities of a few individual VOCs emitted by non-flowering and flowering Dubbele witte market class A plants and flowering Wairimu. BCMV infection induced changes in principal VOCs in non-flowering Dubbele witte market class B, whereas, in flowering plants, the principal VOCs were similar to those of mock-inoculated plants and overlapped. BCMNV infection did not induce significant changes in the principal VOCs in non-flowering and flowering Dubbele witte market class B and flowering Wairimu, but the quantities of some VOCs were either increased or decreased. BCMNV-infected plants also emitted some VOCs that were unique.

These results are consistent with findings by several authors who reported changes in VOC blends induced by virus infection of host plants (Fereres & Moreno 2009; Mauck *et al*. 2010; 2012; Westwood *et al*. 2013a; Groen *et al*. 2016; Tungadi *et al*. 2017; Jiang *et al*. 2017; Bravo 2019; Wamonje *et al*. 2019) (see Section 1.5 in Chapter 1). Wamonje *et al*. (2019) reported varying qualitative and quantitative changes in VOCs emitted by non-flowering *P. vulgaris* cv. Wairimu

induced by BCMV, BCMNC and CMV infection. Groen *et al*. (2016) and Jiang (2017) reported that CMV-infected tomato plants emitted VOC blends that were distinct, and some individual VOCs were either increased or decreased compared to those from mock-inoculated plants.

The biosynthesis of several VOCs is still a grey area. Some of the known pathways, such as the shikimate pathway produce benzaldehyde, among other aldehydes (Dey & Harborne 1997). The mevalonate-independent pathway and the 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP) localized to plastids produces monoterpenoids like linalool (Lichtenthaler *et al*. 1997; Raguso & Pichersky 1999). According to McConkey *et al*. (2000) and Muhlemann *et al*. (2012), the step by step biosynthesis of VOCs is regulated mainly at gene expression level. This is evidenced by synchronized temporal changes in activities of enzymes involved in the final steps of VOCs formation, enzyme protein content, and the expression of corresponding structural genes (McConkey *et al*. 2000; Muhlemann *et al*. 2012). According to Muhlemann and colleagues (2012), transcriptional regulation of VOC-producing biosynthetic pathways is shared by one or multiple intermediate steps, not being limited to their final biochemical step. As suggested by Groen and colleagues (2016), viruses may interfere with small RNA pathways in the biosynthesis of VOCs, which may explain differences in VOC emission by mock-inoculated and virus-infected plants. These key findings may explain how viruses modified the VOC blends in *P. vulgaris* in this current study. There is need for further studies to understand the mechanisms by which viruses alter VOC emissions in hosts.

4.3.2 Viruses alter emission of VOCs known to attract foraging bees

Plants use VOCs to perform a variety of tasks such as defence against herbivorous insects, pollinator attraction, plant-to-plant communication, thermo-tolerance and environmental stress adaptation. The hexenyl family of VOCs and some terpenoids are known to recruit predators of

plant herbivores (Shiojiri *et al*. 2012; Ozawa *et al*. 2013). In flowering BCMNV and CMV-infected cv. Wairimu plants, levels of 3-hexen-1-ol were increased (Table 4.6). VOCs like isoprene and terpenoids contribute to thermotolerance and heat stress mitigation (Singsaas *et al*. 1997; Singsaas & Sharkey 1998; Spinelli *et al*. 2011). VOCs of interest to this study are plant-pollinator interaction regulating ones. These bee-attracting VOCs are limonene, linalool, ocimene, pinene and benzaldehyde (Granero *et al*. 2005; Knudsen *et al*. 2006; Dötterl & Vereecken 2010; Klatt *et al*. 2013; Krug *et al*. 2018). I have shown that virus infection of *P. vulgaris* induces either an increase or decrease in the relative abundance of VOCs that attract foraging bees. BCMV infection significantly decreased the emission of linalool, whereas BCMNV decreased linalool and β-pinene emission in flowering Dubbele witte market class B. In flowering Wairimu, BCMNV infection significantly increased the emission of D-limonene (Table 4.6). Although not statistically significant, some bee attracting VOCs like ocimene and linalool appeared to be decreased in virus-infected cv. Wairimu plants, whereas benzaldehyde appeared to be increased. In flowering cv. Dubbele witte market class B, benzaldehyde, D-limonene, linalool and pinene appeared to be increased, although these were not statistically different from emissions in mock-inoculated plants. Ocimene has been reported to act as a foraging recruitment pheromone in bumblebees (Granero *et al*. 2005). Guarana flowers emit larger quantities of linalool that attract nocturnal foraging bees (Krug *et al*. 2018). I speculate that even the smallest increase or decrease in emission rates of these bee attracting VOCs may affect the foraging behaviour of bees.

Although VOC blends are more important for insect host plant preference than single compounds, increasing emission of bee-attracting VOCs, as observed in increased levels of D-limonene in BCMNV-infected plants, may provide a competitive advantage over lower quantities in overall VOC blends. It has been shown that yellow-faced bumblebees (Byers *et al*. 2014) and black bean aphids (Webster *et al*. 2010) do not respond to individual VOCs but to blends of the VOCs instead. Byers *et al*. (2014) observed that workers of *B. vosnesenskii* preferred a combination of

limonene, myrcene and ocimene over any single compound alone. Reducing the emission of bee-repelling VOCs in overall VOC blends may also provide a competitive advantage for a plant to attract more pollinators. Naïve bumblebees had an innate preference for VOC blends from CMV-infected tomatoes that had lower quantities of bee-repelling 2-carene and β-phellandrene and significantly larger quantities of bee-attracting pinene and cymene (Groen *et al*. 2016). Although virus-induced changes in the emission of most of the bee-attracting VOCs were not statistically significant in this study, some authors like Parachnowitsch and colleagues (2012) suggest that modification of a few key VOCs in floral scent could potentially affect pollinator attraction. This could result in reproductive isolation in nature, but these effects still remain unknown (Parachnowitsch *et al*. 2012). Hence there is need to test the foraging behaviour of bees under controlled conditions in response to specific and mixtures of VOCs changes to identify the biologically important changes induced by virus infection in common bean.

According to Kárpáti *et al*. (2013), foraging hawkmoths consider green leaf VOCs as background to floral blend and use the folia background to their benefit when foraging for rewarding nectar sources. My work showed that typical green-leaf VOCs that were consistently found on both non-flowering and flowering plants were either increased or decreased by virus infection. These are methyl 14-(2-octylcyclopropyl)tetradecanoate, sec-butyl nitrate, nonanal, hexenyl family of VOCs, and 1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate all *P. vulgaris* plants used in this study. In cv. Dubbele witte market class A, benzaldehyde, linalool, and nonanal were emitted in both non-flowering and flowering plants. I speculate that foraging bees will possibly use these altered VOCs in blends as background signals. Groen *et al*. (2016) showed that naïve bumblebees had an innate preference for VOC blends from both non-flowering and flowering plants. Groen *et al*. (2016) suggested that green-leaf VOCs may play an essential role in attracting bees from a distance, synergizing with and reinforcing visual clues, as observed with floral scent (Kulahci *et al*. 2008; Kunze & Gumbert 2001).

CHAPTER 5

BUMBLEBEES HAVE AN INNATE PREFERENCE FOR VOLATILE ORGANIC COMPOUND BLENDS EMITTED BY VIRUS-INFECTED COMMON BEAN PLANTS

5.1 INTRODUCTION

Bumblebee foraging behaviour is often affected by floral scent (Kunze & Gumbert 2001, Laloi & Pham-Delegue 2004). Bumblebees have a highly developed sense of smell; they possess more than 130 different olfactory receptors in their antennae (Chittka & Raine 2006). It has been hypothesized that olfactory cues play a more critical role in differentiating specific host plant species, signalling the appropriate phenological stage for pollinator visits and attracting pollinator to rewardless sex in dioecious plant species, compared to visual cues (Chen *et al.* 2009; Hossaert-McKey *et al.* 2010; Song *et al.* 2014). According to Raguso (2008), bees exhibit innate preferences for certain floral odours. The effects of non-floral VOCs and a combination of floral and non-floral VOCs (as with flowering plants) on naïve bumblebees foraging behaviour are less well understood. Experiments conducted in our laboratory demonstrated that buff-tailed bumblebees have an innate preference for VOC blends emitted by non-flowering and flowering CMV-infected tomato plants (Groen *et al*. 2016; Jiang 2017). In this section of my study, I extended this work using *P. vulgaris* as the host plant and three viruses: CMV, BCMV and BCMNV. In the previous chapter, I showed that viruses alter the VOC blends emitted by *P. vulgaris* cvs. Dubbele witte and Wairimu. The objective of this section was to investigate whether the innate preference of bumblebees for VOC blends emitted by virus-infected plants is unique to the tomato-CMV pathosystem or is widespread across different hosts and viruses.

5.2 RESULTS

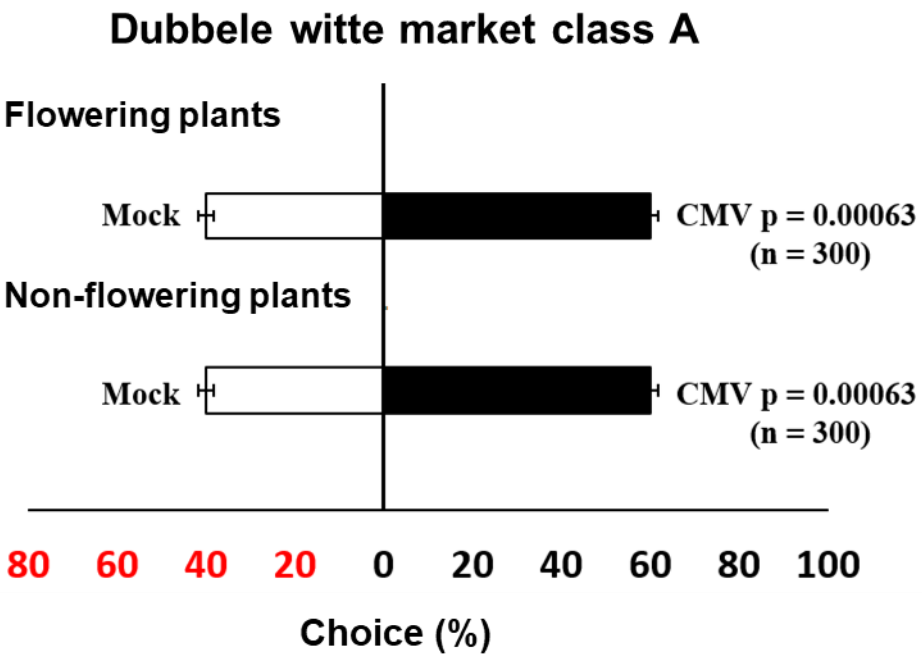
5.2.1 Bumblebees showed an innate preference for VOC blends emitted by virus-infected plants

In free-choice assays, bumblebees were released one at a time into flight arenas containing ten common bean plants (five mock-inoculated and five virus-infected plants), concealed within towers designed to allow VOC blends to diffuse out but prevent the bees from seeing or touching the plants (Figure 2.6B). Approximately 50 µl of 30 % (w/v) sucrose reward was placed in each micro-centrifuge tube lid attached to the towers concealing plants of both treatment groups to encourage bumblebees to feed from several towers before returning to the colony (Figure 2.6B). The lids were refilled when the bee went back to the colony, and the towers were wiped with 30% v/v ethanol to remove scent marks and re-arranged randomly to prevent spatial learning. Bumblebees preferred to visit the towers that were hiding virus-infected plants when presented with both non-flowering and flowering mock-inoculated and BCMV and CMV-infected plants (Figure 5.1). The bumblebees responded differently to VOC blends from BCMNV-infected plants. They preferred to visit towers concealing non-flowering BCMNV-infected plants over mock-inoculated ones but showed no difference in preference for visiting towers that concealed flowering BCMNV-infected plants and mock-inoculated plants (Figure 5.1B & C). This indicates that bumblebees have an innate preference for VOC blends emitted by non-flowering and flowering common bean plants infected with BCMV and CMV. The data also show that flowering BCMNV-infected plants lose the attraction they had before flowering.

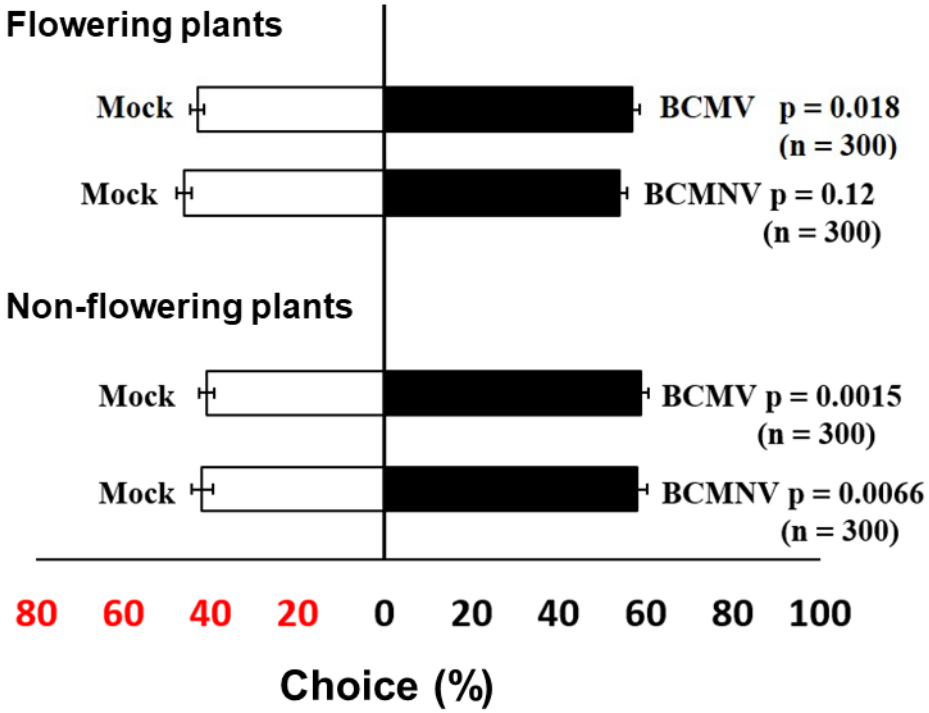
5.2.2 Bumblebees can discriminate between VOCs emitted by flowering BCMNV-infected and mock-inoculated plants

Differential conditioning was used to determine if bumblebees could perceive the differences in VOC blends emitted by flowering BCMNV-infected and mock-inoculated plants. This was done because, in free choice assays, bumblebees showed no innate preference for or against VOC blends emitted by flowering BCMNV-infected plants. I wanted to test whether this was because the bees were unable to perceive the difference(s) or could perceive difference(s) in VOCs but

A


Dubbele witte market class A
Flowering plants
Mock
CMV p = 0.00063
(n = 300)
Non-flowering plants
Mock
CMV p = 0.00063
(n = 300)
80 60 40 20 0 20 40 60 80 100
Choice (%)

B


Dubbele witte market class B
Flowering plants
Mock
BCMV p = 0.018
(n = 300)
Mock
BCMNV p = 0.12
(n = 300)
Non-flowering plants
Mock
BCMV p = 0.0015
(n = 300)
Mock
BCMNV p = 0.0066
(n = 300)
80 60 40 20 0 20 40 60 80 100
Choice (%)

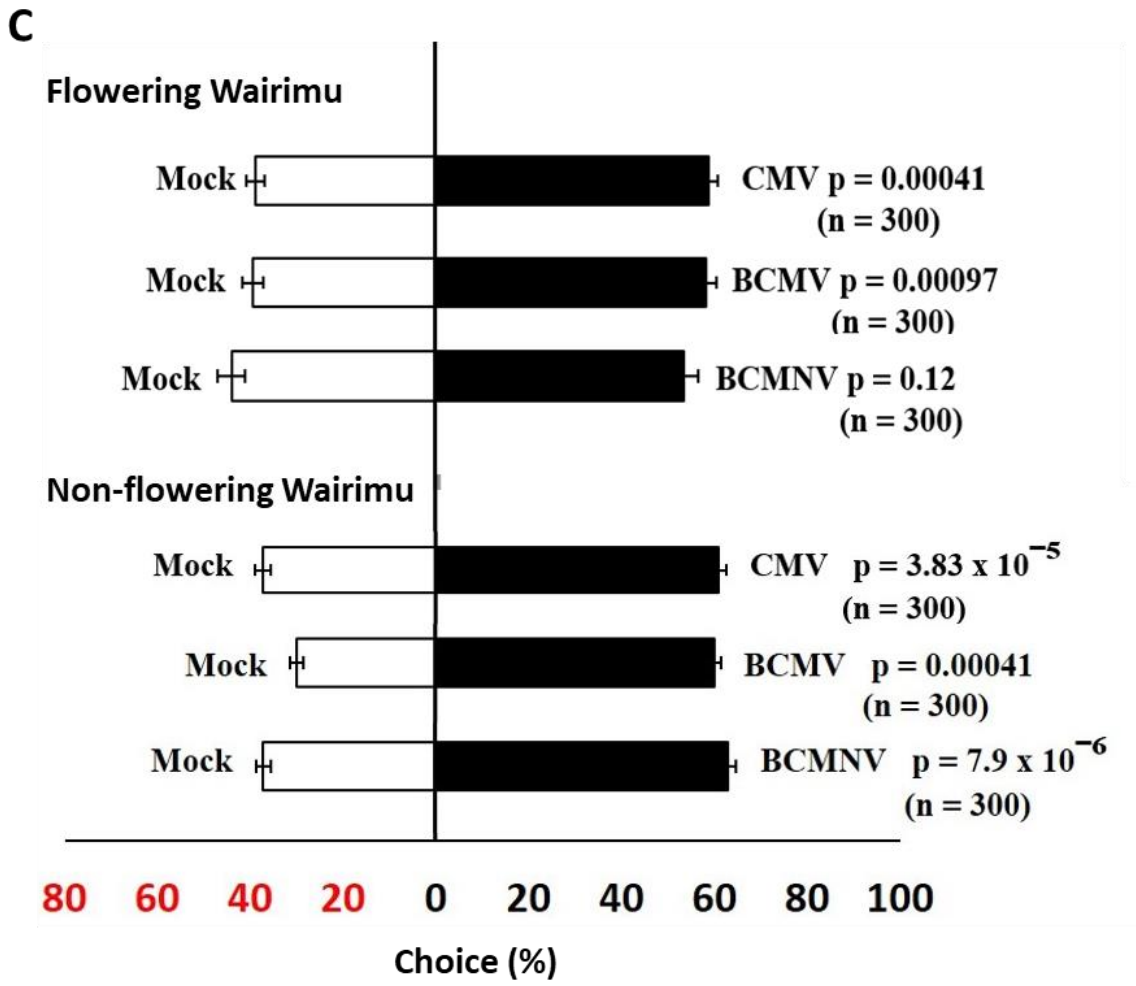


Figure 5.1. Bumblebees prefer VOC blends emitted by virus-infected common bean plants. In free-choice assays, VOC blends from virus-infected plants attracted more visits by bumblebees than those from mock-inoculated plants (A, B, & C). However, bumblebees showed no preference for flowering BCMNV-infected cv. Dubbele witte (B) and cv. Wairimu (C). Non-flowering virus-infected plants strongly attracted bumblebees than flowering virus-infected plants did. The p-values shown in each panel are from binomial tests of pooled data of the first 10 choices of 30 naïve bumblebees in A, B and C. Error bars represent the standard error of the mean.

had no preference. Flowering plants from each treatment were covered by black feeding towers to eliminate visual and tactile cues (see Section 2.7.3). Five flowering mock-inoculated plants were paired up with 50 µl of 30 % (w/v) sucrose reward, and five flowering BCMNV-infected plants with 50 µl quinine hemisulphate solution (0.12% w/v) as punishment (Section 2.7.3). Bumblebees were released one at a time and the first 100 choices of each bee recorded. In both flowering cv. Dubbele witte (Figure 5.2A) and cv. Wairimu (Figure 5.2B), the bees showed a significant learning behaviour increasingly avoiding towers concealing flowering BCMNV-infected plants as they foraged. By the 100th choice, the bees had reached an 80 % or greater accuracy of associating sucrose reward with its assigned olfactory stimulus – VOC blends from flowering mock-inoculated plants (Figure 5.2 A and B). This shows that the bees can distinguish between the VOC blends of flowering mock-inoculated and BCMNV-infected plants.

5.2.3 Bumblebees show no innate preference for VOC blends from either non-flowering or flowering mock-inoculated plants.

I went on further to test whether bumblebees preferred VOC blends from non-flowering plants or flowering plants in mock-inoculated plants. Free-choice assays were used, offering the bumblebees equal rewards of 50 µl of 30 % (w/v) sucrose reward on towers covering five non-flowering mock-inoculated plants and five flowering mock-inoculated plants. I used cv. Wairimu variety in these experiments. Towers covering both two groups of plants were equally visited by bees meaning naïve bumblebees do not have an innate preference for VOC blends emitted by non-flowering and flowering mock-inoculated cv. Wairimu plants (Figure 5.3). These results suggest that green leaves rather than flowers are the primary source of bee-attracting VOCs.

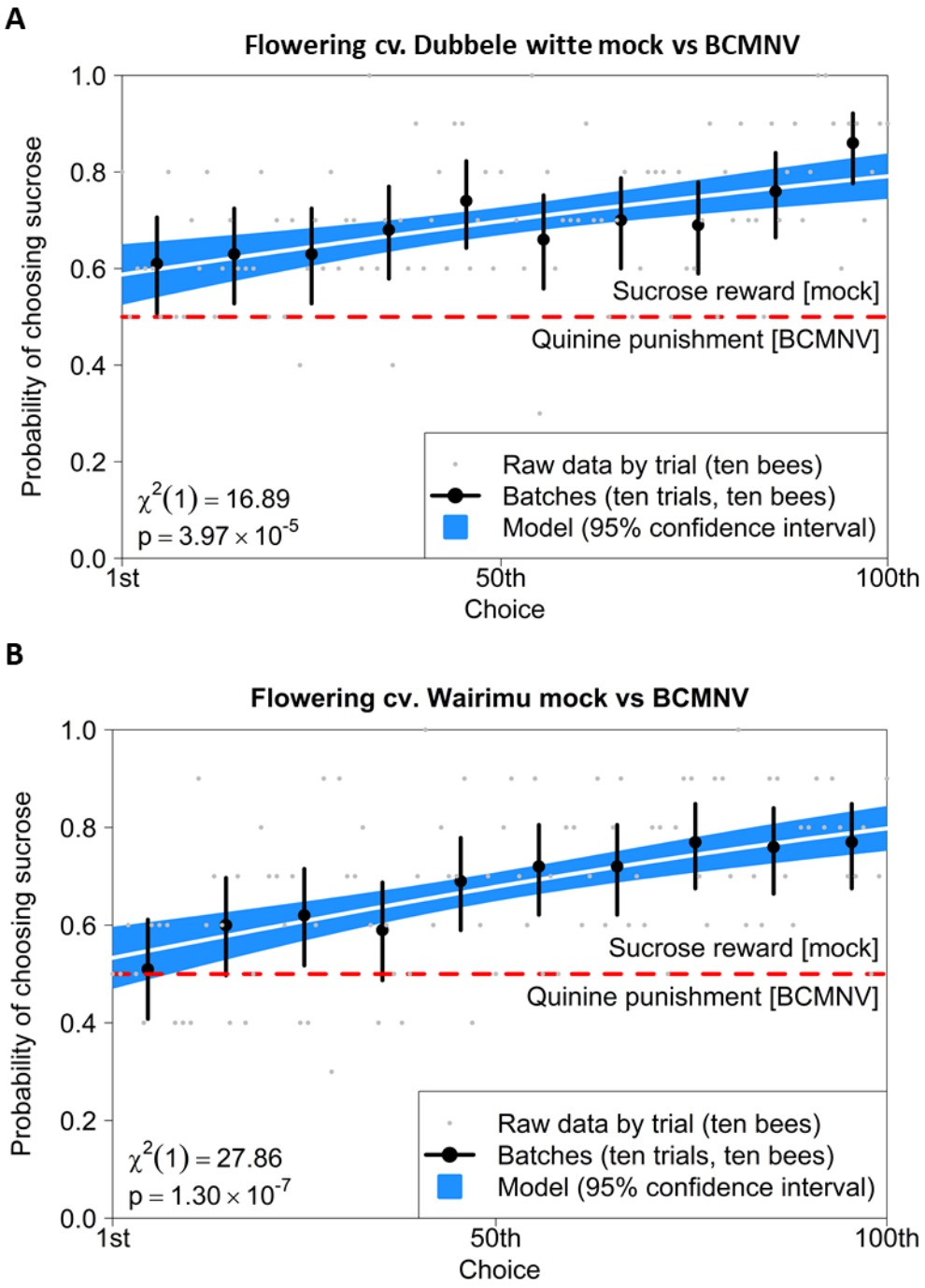


Figure 5.2 Bumblebees can learn to distinguish between flowering mock-inoculated and BCMNV-infected plants. Bumblebees can be trained by differential conditioning (using 30% sucrose-reward and 0.12% quinine punishment) to distinguish between VOC blends emitted by flowering common bean. The learning curve indicates the overall ability to distinguish between plant-emitted volatiles analyzed after 100 choices per bee using 10 bees. It was determined

bumblebees could perceive the differences between volatiles emitted by flowering mock-inoculated plants and BCMNV-infected plants. In flowering cv. Dubbele witte bean market class B (A), bumblebees started off by choosing sucrose reward at an average accuracy of 60% in their first 10 choices. By choices 90 to 100, their average accuracy increased to about 85%, as indicated by a rising learning curve, meaning bumblebees learnt to identify sucrose rewards based on the association with volatiles from flowering mock-inoculated plants. Similar results were obtained with flowering cv. Wairimu (B), the bumblebees learnt to associate sucrose reward with VOC blends from flowering mock-inoculated plants. Initially in the first 10 chose 50% sucrose at an average accuracy of 50%. This increased to an average of about 75% by choices 90 to 100. Data are shown pooled over all bees (n = 10) into successive groups of 10 choices, with error bars showing 95% binomial confidence intervals for the proportion of correct choices. The white curve shows the fitted binomial logistic model, with blue borders showing 95% confidence intervals on the fitted response. The statistic and p-value for the likelihood ratio test assessing whether bees are able to learn are given at the bottom left of each panel.

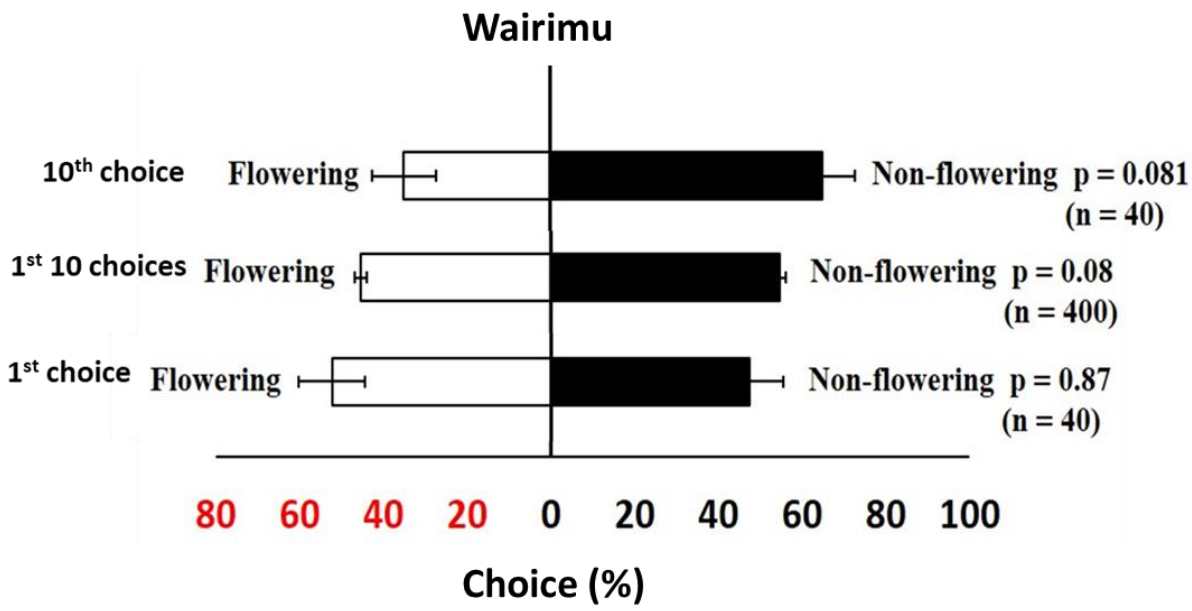


Figure 5.3 VOC blends of non-flowering and flowering mock-inoculated Wairimu are equally appealing to bumblebees. In free-choice assays, naïve bumblebees showed no preference for either non-flowering or flowering mock-inoculated cv. Wairimu plants. Towers covering flowering and non-flowering plants were visited almost equally by naïve bumblebees. The p-values shown in each panel are from binomial tests of the means of the first choice of the 40 bees (lower panel), pooled data of the first 10 choices of 40 naïve bumblebees (middle panel) and the 10th choice of the 40 bees. Error bars represent the standard error of the mean.

Table 5.1 Summary of results from free choice and differential conditioning assays

Treatment	BCMV	BCMNV	CMV
Free choice assays with cv. Dubelle wite			
Non-flowering plants	Bumblebees showed an innate preference for VOCs	Bumblebees showed an innate preference for VOCs	Bumblebees showed an innate preference for VOCs
Flowering plants	Bumblebees showed an innate preference for VOCs	Bumblebees had no innate preference for VOCs	Bumblebees showed innate preference for VOCs
Free choice assays with cv. Wairimu			
Non-flowering plants	Bumblebees showed an innate preference for VOCs	Bumblebees showed an innate preference for VOCs	Bumblebees showed an innate preference for VOCs
Flowering plants	Bumblebees showed an innate preference for VOCs	Bumblebees had no innate preference for VOCs	Bumblebees showed innate preference for VOCs
Differential conditioning assays			
Flowering cv. Dubbele witte	N/A	Bumblebees could differentiate VOCs from those of mock-inoculated plants	N/A
Flowering cv. Wairimu	N/A	Bumblebees could differentiate VOCs from those of mock-inoculated plants	N/A

5.3 DISCUSSION

5.3.1 Naïve bumblebees have an innate preference for VOC blends emitted by common bean plants infected with three different viruses

I have shown that bumblebees have an innate preference for VOC blends emitted by non-flowering and flowering BCMV and CMV-infected common bean plants (Table 5.1). The bees also had an innate preference for non-flowering BCMNV-infected plants, but upon flowering, the innate preference was lost. In Chapter 4, I showed that BCMV, BCMNV and CMV infections all induce qualitative and quantitative changes in VOC blends emitted by common bean plants. According to Parachnowitsch and colleagues (2012), modification of a few key VOCs in floral scent could potentially affect pollinator attraction and reproductive isolation in nature, but these effects still remain unknown. The ability of viruses to modify the VOCs quantitatively and qualitatively appears to affect the choices of bees. As previously observed by Groen and colleagues (2016), viruses modify VOC blends in such a way that they elicit innate preference in bumblebees for the VOCs emitted by their hosts.

In flowering BCMNV-infected common bean, the innate attraction of buff-tailed bumblebees to VOCs blends is lost, but very strong in non-flowering plants. BCMNV-infection induces significant changes in principal VOCs in non-flowering and flowering plants, as these two developmental stages have principal VOCs that cluster separately in a principal component analysis (Figure 4.18C, Chapter 4). Given the virulence and observed severity of symptoms caused by BCMNV that increasingly worsen as the plant ages, (personal observations and see Chapter 3 Figure 3.1), modification of VOCs that attract bees in flowering plants might be greatly compromised by virus accumulation. The bumblebees showed no innate preference for VOCs from non-flowering and flowering mock-inoculated plants, meaning the blends from these two developmental stages of common bean have principal VOCs that are almost equally attractive to bees as observed in the

previous chapter (Figure 4.17A & 4.18A). These results suggest that olfactory cues from green leaves may play a more significant role in attracting bees at long ranges than previously thought.

VOC blends have been suggested to be important cues for naïve bees in search of their first floral meal or to help bees find new foraging patches or food sources (Roy & Raguso 1997; Dötterl *et al*. 2005) and may aid bees foraging in poorly lit habitats (Knudsen *et al*. 1999). VOCs may function as long and/or short-distance attractants (reviewed by Dobson 1994). Experienced bees utilize floral scent cues to recognize previously visited flowers and learn to associate rewarding flowers with olfactory cues (Dobson & Bergstrom 2000; Howell & Alarcon 2007; Arenas & Farina 2012; Wright & Schiestl 2009). This leads to the flower constancy phenomenon whereby individual bees will exclusively visit certain flower species or morphs within a species, sidestepping other available flower morphs or species that could be potentially more rewarding (Chittka *et al*. 1999; Raine *et al*. 2006; Wright & Schiestl 2009).

This implies that in natural environments, naïve bees will initially visit virus-infected plants. If virus-infected plants are more rewarding than healthy plants, then the preference will be reinforced through learning, and the infected plants will continue to be visited by experienced bees. It was noted that under lower light conditions, virus infection induced the production of more concentrated nectar in larger volumes in cv. Wairimu (see Chapter 3, Section 3.2.6). As noted above, VOC cues are more effective in lower light conditions. Thus, viruses make host plants more attractive to their pollinators using VOCs to improve floral visitation and ultimately increased yield. A similar preference for VOCs of CMV-infected tomatoes was noted by Groen *et al*. (2016). My studies show that the innate preference for VOC blends emitted by virus-infected plants is not unique to the CMV-tomato pathosystem. Indeed, my work shows that this phenomenon occurs in different plant hosts with a different pollination syndrome from tomato and that it can be induced by viruses other than CMV.

5.3.2 Bumblebees can discriminate between volatile profiles from flowering mock-inoculated and BCMNV-infected common bean

Differential conditioning using quinine as a punishment is a well-established technique used to assess the cognitive ability of bees to perceive and learn floral cues such as olfactory, visual and tactile cues (Arenas *et al*. 2009; Whitney *et al*. 2009; Avarguès -Weber *et al*. 2010; Reser *et al*. 2012; Groen *et al*. 2016). I have shown that bumblebees can distinguish VOC blends emitted by flowering BCMNV-infected plants from those flowering of mock-inoculated plants, using differential conditioning. The bumblebees had shown no preference for VOC blends from neither flowering BCMNV-infected nor mock-inoculated plants. The bees were then able to associate VOCs from flowering mock-inoculated plants with rewarding 30 % sucrose solution and quinine punishment with VOCs from flowering BCMNV-infected plants.

Although the principal VOCs in blends from cv. Wairimu these two treatments showed some similarities through overlapping in PCA (Figures 4.4B & 4.7), the ratios of individual VOCs differed (Figures 4.5 & 4.8). For foraging bees, specific VOC identities and ratios are necessary for perception of the scent (Wright *et al*. 2005; Najar-Rodriguez *et al*. 2010). Hence the bumblebees could perceive the differences. The specific VOC ratios differed between flowering mock-inoculated and flowering BCMNV-infected plants in the blends. Thus by association with VOC blends, the bees then learnt not to forage from towers covering flowering BCMNV-infected plants because they offered quinine punishment. My results are consistent with those of Groen *et al*. (2016), who demonstrated that buff-tailed bumblebees could discriminate VOC profile from mock-inoculated tomato and tomato plants infected with CMVΔ2b - a mutant form of CMV unable to express the 2b viral suppressor protein using differential conditioning. The bees had shown no innate preference for either mock-inoculated or CMVΔ2b-infected tomato plants (Groen *et al*. 2016). As explained in Section 1.8, the 2b protein plays an essential role in regulating the emission of bee-perceivable VOCs in CMV-infected plants. In future, similar experiments could be done in

common bean with the mutant CMVΔ2b to ascertain that viral proteins reprogram VOCs in hosts to attract pollinators.

CHAPTER 6

VIRUS-INDUCED POLLINATOR ATTRACTION PAYS BACK INFECTED PLANTS WITH IMPROVED REPRODUCTIVE FITNESS

6.1 INTRODUCTION

In this chapter, I report the results from experiments done to interrogate further the 'payback' hypothesis and determine whether virus infection conferred any reproductive advantage to bee-pollinated hosts through induced pollinator attraction. I hypothesized that viruses pay back plant hosts by attracting pollinators which causes yield recovery in their susceptible partially pollinator-dependent hosts, *P. vulgaris*. This is an extension of previous landmark studies by Groen and colleagues (2016) who proposed the payback hypothesis. I used a different phytosystem, a plant host with a more complex flower, different pollination system and different reward to pollinators than tomato plants used by Groen and colleagues (2016). Tomatoes are buzz pollinated while beans are trip pollinated. Also, while the reward from tomato plants to pollinators is pollen, bean flowers offer nectar rewards with the pollen reward as a bonus.

The model pollinator *B. terrestris* was used in these experiments amongst virus-infected and mock-inoculated flowering common bean plants under controlled (glasshouse) conditions and natural environments in the University of Cambridge Botanic Garden where other wild bee pollinators are existent. The effect of virus infection on bee-mediated reproductive success was determined by comparing seed production in pods from in mock-inoculated and virus-infected bean plants that had either been trip-pollinated or not trip-pollinated. In the glasshouse, experiments were done using cv. Dubelle witte market classes A and B plants with CMV and cv. Wairimu with BCMV and CMV. In the Botanic garden, experiments were done using the three

varieties. Cv. Dubelle witte market class B plants with CMV were raised in the garden, whereas potted Dubelle witte market class A plants with CMV were raised in the glasshouse and translocated to the garden upon flowering. Cv. Wairimu plants with BCMV were raised in the garden in a separate plot from cv. Dubelle witte market class B plants with CMV. BCMNV could not be used in the glasshouse and Botanic garden because it is a licensed pathogen only to be used under strictly contained conditions.

6.2 RESULTS

6.2.1 Bumblebees showed an increasing preference for CMV-infected cv. Dubbele witte market class A and mock-inoculated cv. Wairimu over the course of free choice assays

To investigate the effects of virus infection on bee pollination behaviour in common bean plants under glasshouse conditions, bumblebees were allowed to freely pollinate 6 mock-inoculated and 6 virus-infected plants in a large flight arena (as described in Section 2.8, Figure 2.7). Due to time limitations and pest infestation, I was able to complete experiments with CMV infection in cv. Dubbele witte market class A and cv. Wairimu and experiments with BCMV infection in cv. Wairimu only.

In cv. Dubbele witte market class A, 57% of the bumblebees made their first visits to flowers of mock-inoculated plants (Figure 6.1A & C). From the second to the tenth choice, a higher proportion of the bees visited flowers on CMV-infected plants (Figure 6.1A & C). When the first 10 choices of the 30 bees were pooled together and analysed, the preference for flowers on CMV-infected plants over flowers of mock-inoculated plants was significant (Figure 6.1C). The bees' preference for visiting flowers on CMV-infected plants increased over the first 10 choices (Figure 6.1B).

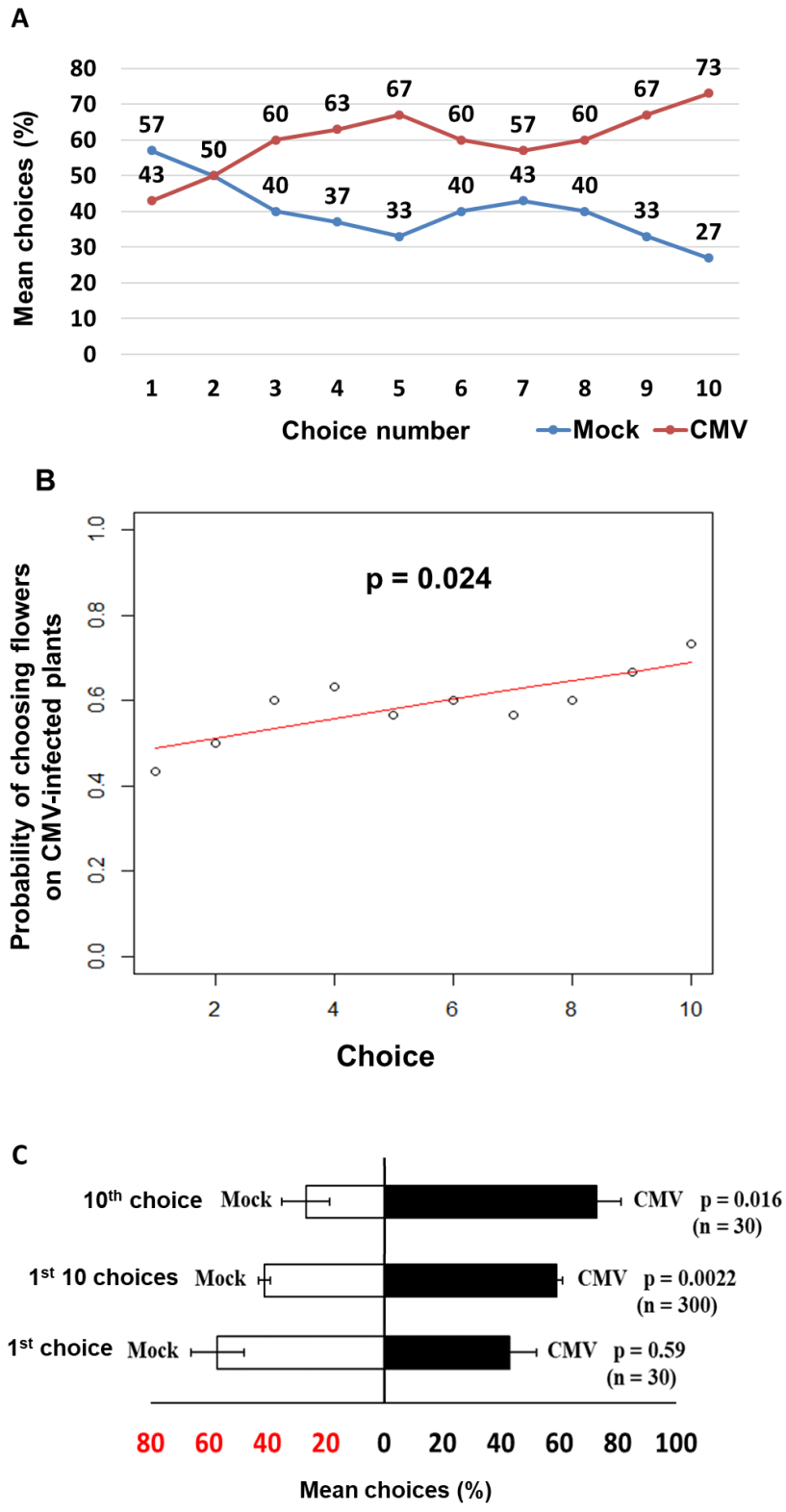


Figure 6.1 First 10 choices of 30 bumblebees on flowers of cv. Dubbele witte market class A plants under glasshouse conditions. Free foraging bumblebees made their first visit on

flowers of mock-inoculated plants. From the second to the tenth choice, the bees increasingly visited flowers of CMV-infected plants (A). This suggests a significant degree of learning behaviour as the probability of choosing flowers of CMV-infected plants increased over their first 10 choices (B). (C) When the first 10 choices of 30 bumblebees were pooled together, the preference for flowers of CMV-infected plants was statistically significant (middle panel). By the tenth choice, the bees made significantly more visits to flowers of CMV-infected plants. The levels of significance are shown by p-values calculated with binomial tests of pooled data of the first 10 choices of 30 naïve bumblebees. The red curve in (B) shows the fitted binomial logistic model and dots are means of raw data per choice. Error bars in (C) represent standard error of the mean.

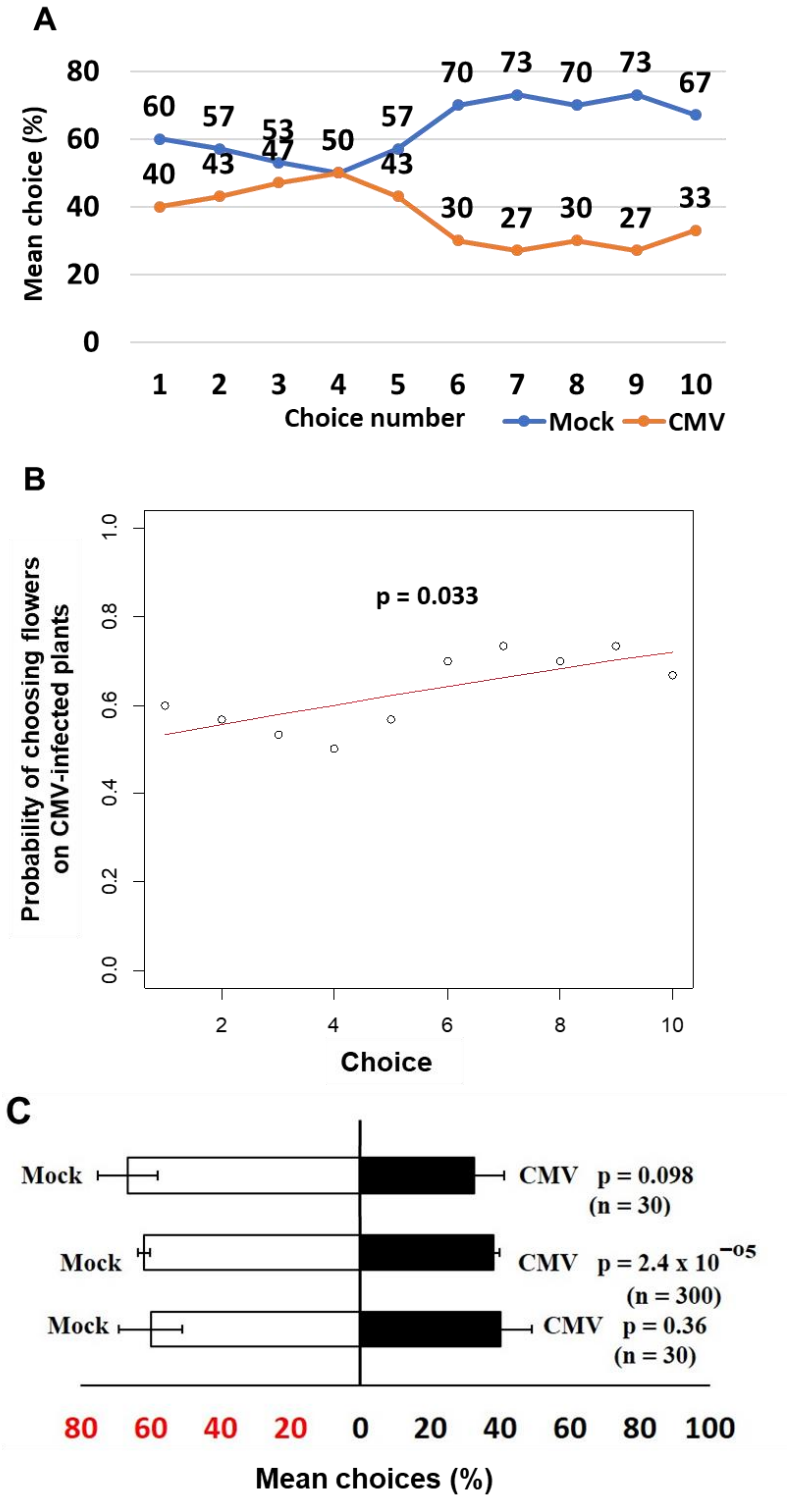


Figure 6.2 First 10 choices of 30 bumblebees foraging on flowers of cv. Wairimu plants under glasshouse conditions. The first choice of 60% of the free foraging bumblebees was on flowers on mock-inoculated plants, as shown in (A) and (C). (A) From the second through the

fourth choice, the number of visits to flowers on mock-inoculated plants decreased. Then from the fifth to the tenth choice, the bumblebees increasingly visited flowers on mock-inoculated flowers. (B) The bumblebees showed an increasing bias towards choosing flowers on mock-infected plants increased over their 10 choices, which is suggestive of learning behaviour. (C) The preference for flowers on mock-inoculated plants was significant (middle panel). When the first 10 choices of 30 bumblebees were pooled together, the bees made significant visits to flowers on mock-inoculated plants. The levels of significance are shown by p-values calculated with binomial tests. The red curve in (B) shows the fitted binomial logistic model and dots are means of raw data per choice. Error bars in (C) represent standard error of the mean.

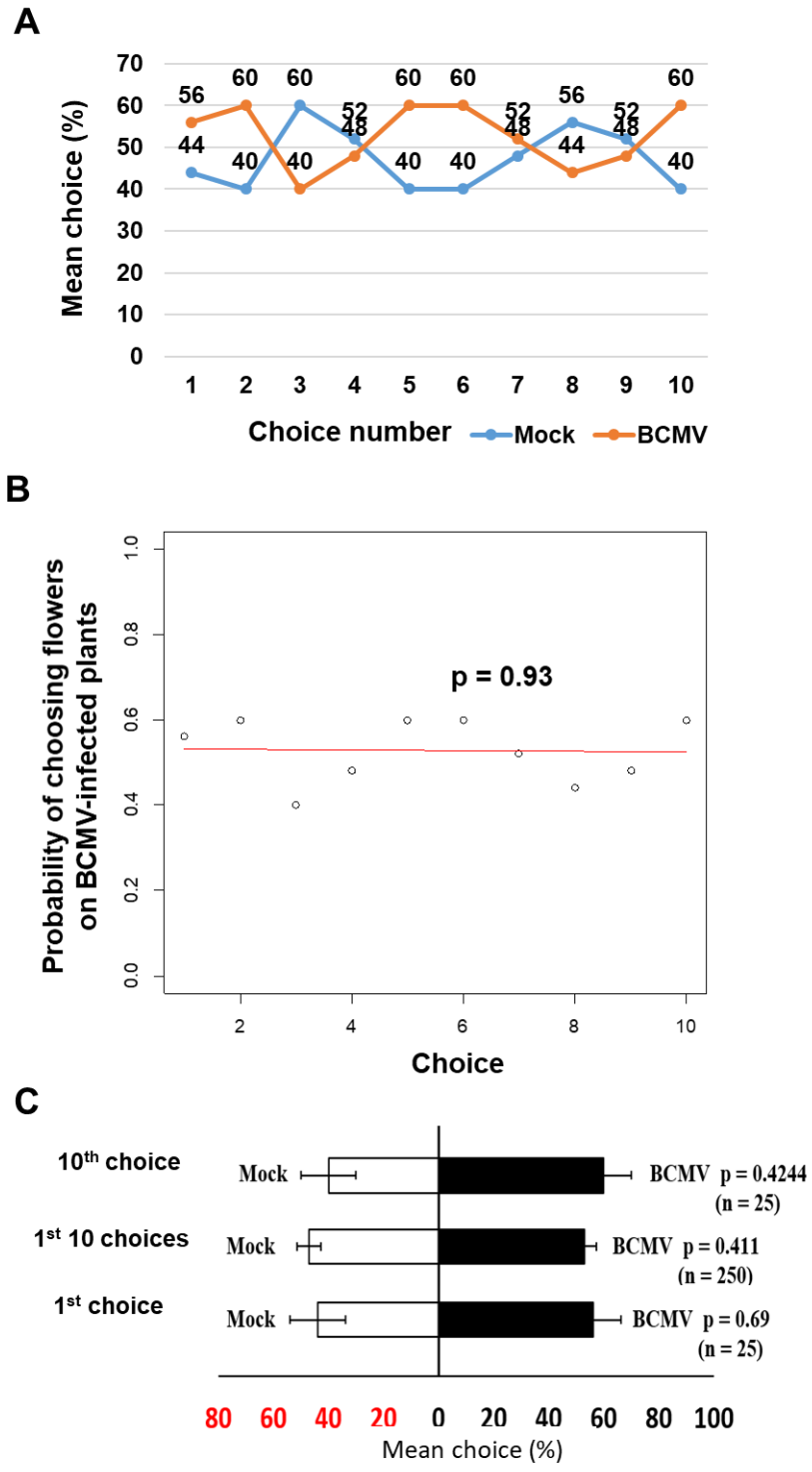


Figure 6.3 First 10 choices of 25 bumblebees foraging on flowers of cv. Wairimu plants under glasshouse conditions. Bumblebees showed no preference for flowers of either mock-inoculated or BCMV-infected plants throughout their first 10 visits (A & C). Neither did they show

any evidence of preference for flowers of any of the two treatments (B). The levels of significance are shown by p-values calculated with binomial tests. The red curve in (B) shows the fitted binomial logistic model and dots are means of raw data per choice. Error bars in (C) represent standard error of the mean.

In flowering cv. Wairimu when bumblebees were allowed to forage on both mock-inoculated and CMV-infected plants, 60% of the bumblebees made their initial visits to flowers on mock-inoculated plants (Figure 6.2A & C). The bees seemed to assess the reward status of the flowers from the two treatments as from the second to the fourth choice because visits to mock-inoculated flowers were decreasing while increasing visits on CMV-infected plants (Figure 6.2A & B). By the fifth visit, the bees increasingly preferred to visit flowers on mock-inoculated plants (Figure 6.2A & B). By the tenth choice, the bees were favouring flowers on mock-inoculated plants. An analysis of the first 10 choices of 30 bees pooled together showed that the bumblebees preferred to visit flowers from mock-inoculated cv. Wairimu (Figure 6.2C).

In experiments with flowering mock-inoculated and BCMV-infected cv. Wairimu plants, bumblebees did not show any preference in visitation on either mock-inoculated plants flowers or BCMV-infected plants flowers. (Figure 6.3C). There were variations in choices made by bumblebees whereby one treatment group seemed to be visited more than the other from the first choice to the tenth choice (Figure 6.3A), but taken together, no treatment group was visited more frequently than the other (Figure 6.3C). The bees did not exhibit any changes in preference (Figure 6.3B).

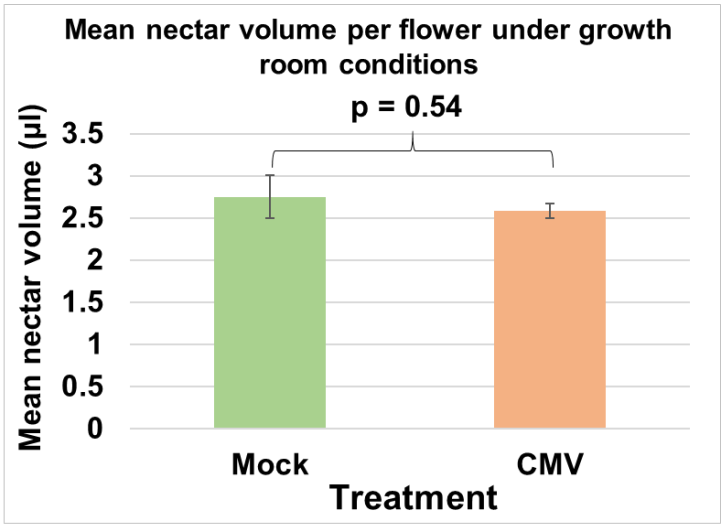
The pollination behaviour of bumblebees on these two varieties of common bean prompted me to assess the nectar rewards in cv. Dubbele witte market class A. I had only assessed floral traits and nectar rewards in cv. Wairimu. It was clear that the naïve bumblebees were no longer exhibiting innate preferences for VOCs as seen in Chapter 5 whereby feeding towers offered an equal reward and the bees had to rely on innate preference for VOCs to make their choices. In the glasshouse flight arena, the bees were using visual, olfactory and tactile cues to choose which flowers to visit. These cues were then associated with reward from flowers of the two treatments and the bees were frequently visiting more rewarding flowers. It was already known that in cv.

Wairimu under glasshouse conditions, there was no difference in nectar quantity in flowers of both mock-inoculated and CMV-infected plants (Figure 3.10, Section 3.2.6, Chapter 3). There was a significant decrease in nectar sucrose concentration in flowers from CMV-infected plants (Figure 3.10, Section 3.2.6, Chapter 3). Flowers of mock-inoculated and BCMV-infected cv. Wairimu offered the same nectar reward (Figure 3.10, Section 3.2.6, Chapter 3). Using the same procedure of nectar extraction and measurements as described in Section 2.5.4 of Chapter 2, it was determined that in cv. Dubbele witte market class A under controlled growth conditions (Section 2.2.1.2, Chapter 2), nectar production and nectar sucrose concentration were similar in both mock-inoculated and CMV-infected plants (Figure 6.4). Under glasshouse conditions, CMV infection induced an increase in nectar production and nectar sucrose concentration (Figure 6.5). These results suggested that the reason bumblebees were learning to visit more rewarding flowers over their first 10 choices was increased nectar reward (Figure 6.1).

6.2.2 Bumblebee pollination rescues seed production in CMV-infected *Phaseolus vulgaris* under controlled glasshouse conditions.

After pollination experiments whereby bumblebees freely pollinated plants of their choice in the large flight arena with 6 mock-inoculated and 6 virus-infected plants, all the flowers that were visited by bees were tagged (as described in Section 2.8, Figure 2.7). All plants were left to grow until their pods were mature. Pods were harvested and categorized into three different groups as seeds from (i) flowers visited by bees (flowers that were visited by bees during pollination experiments and tagged); (ii) flowers not visited by bees (flowers not visited by bees during pollination experiments, and (iii) un-touched flowers (flowers from plants not used in pollination experiments and treated as control plants) (Section 2.8, Chapter 2). Seed numbers from each individual pod were counted and recorded as previously described in Section 2.8.

A



B

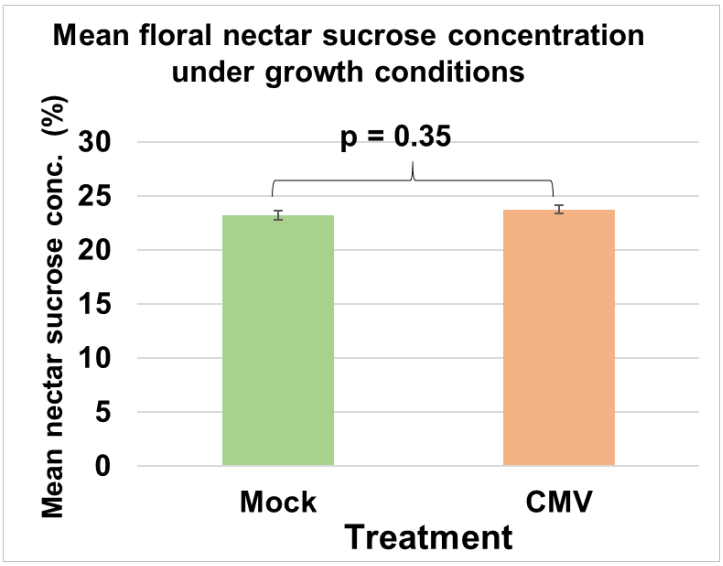


Figure 6.4 CMV did not induce any changes in nectar production in cv. Dubbele witte market class A under controlled growth conditions. There were no significant differences in nectar volume (A) (t = 0.61; df = 198) and nectar sucrose concentration (t = 0.93; df = 198) (B) in Dubbele witte market class A flowers from mock-inoculated and CMV-infected plants grown in controlled growth rooms (Section 2.2.1.2, Chapter 2). Error bars represent the standard error of the mean. The p-values shown are from two-sample t-tests.

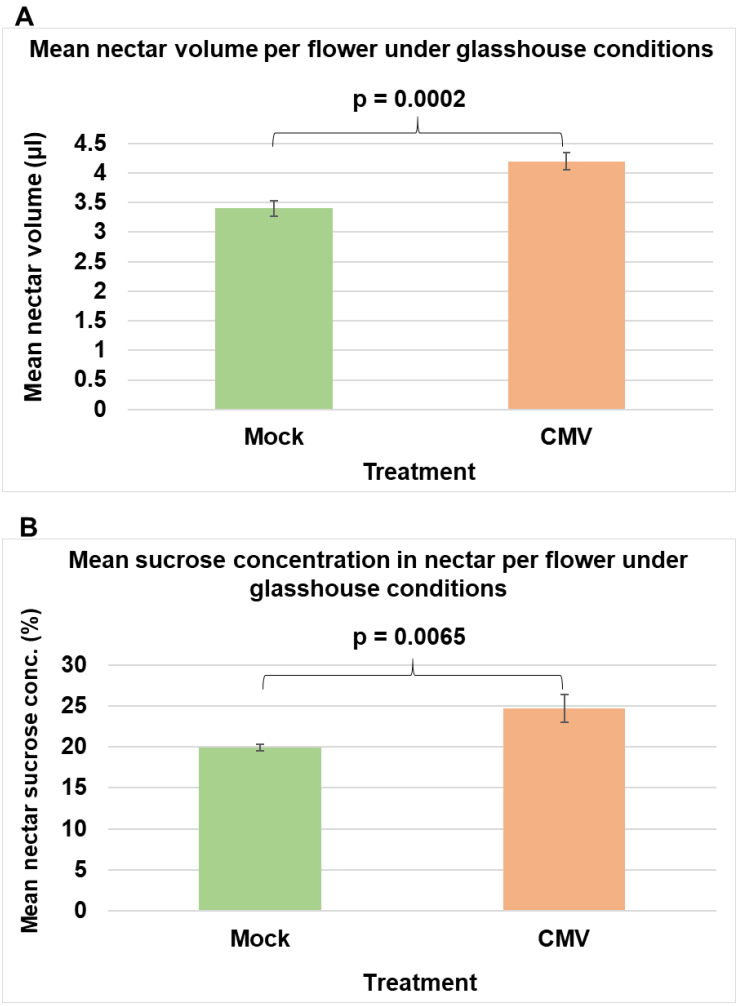


Figure 6.5 CMV infection induced an increase in nectar production in cv. Dubbele witte market class A under glasshouse conditions. When cv. Dubbele witte market call A plants were grown in the glasshouse, CMV-infected plants produced flowers that had (A) increased nectar volume and (B) increased nectar sucrose concentration when compared to flowers of mock-inoculated plants. Error bars represent the standard error of the mean. The p-values shown are from two-sample t-tests (A) t = 3.79; df = 198 and (B) t = 2.75; df = 198.

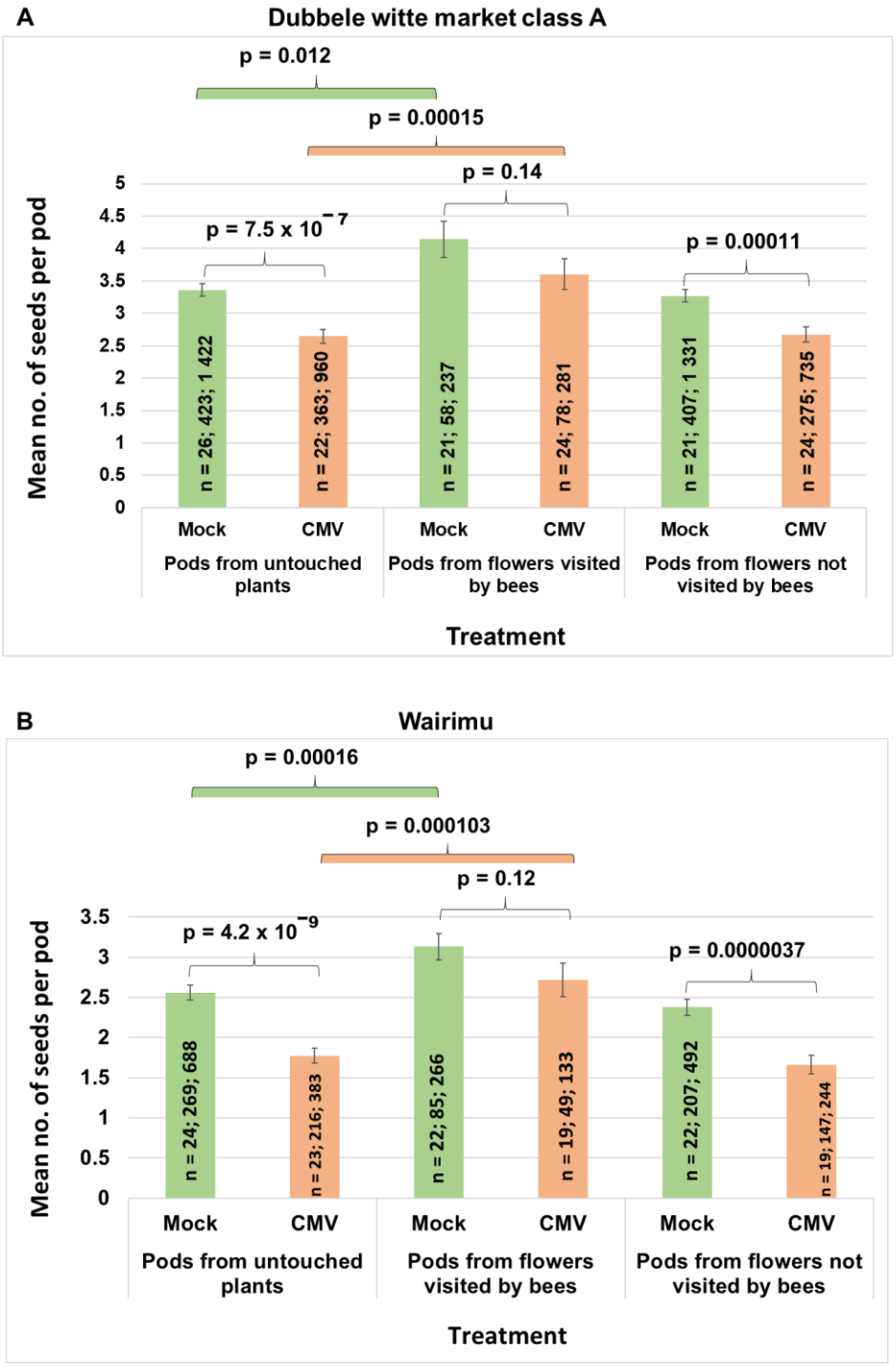


Figure 6.6 Bumblebee pollination compensates for seed yield loss in CMV-infected *P. vulgaris* plants. In the glasshouse, free foraging bumblebees in a flight arena pollinated both mock-inoculated and CMV-infected common bean plants. Flowers visited by bees were marked. The plants were allowed to grow to pod maturity. During harvesting, pods were categorized

according to whether they developed from flowers that were not trip-pollinated by bumblebees (pods from flowers not visited by bees), flowers that were trip pollinated (pod from flowers pollinated by bees), and control pods from flowers that were never exposed to bees, but placed in the flight arena and experienced the same growth conditions as other plant categories (pods from untouched plants). (A) In Dubbele witte market class A, CMV infection significantly reduced the number of seeds produced per pod by 21% (two-sample t-test; t = 4.99; df = 647) in control plants that were not exposed to bumblebees. (B) In control plants of cv. Wairimu not exposed to bumblebees, the number of seeds per pod in CMV-infected plants was significantly lower by 31% (two-sample t-test; t = 5.93; df = 483). (A & B) (A & B) Trip-pollination by bumblebees increased seed production per pod in both mock-inoculated and CMV-infected plants. Interestingly, trip pollination was more beneficial to CMV-infected plants because the seed production matched that of untouched mock-inoculated plants. The p-values shown are from unpaired two-sample t-tests. Histogram bar labelling: n = number of plants; number of pods; number of seeds. Error bars are standard errors around the mean number of seeds.

In both varieties of *Phaseolus vulgaris* used, trip-pollination by bumblebees produced pods that had significant increases in numbers of seeds (Figure 6.6). More interestingly, the number of seeds in pods resulting from flowers visited by bumblebees in CMV-infected plants was not significantly different from those from flowers visited by bees in mock-inoculated plants (Figure 6.6). Flowers from CMV-infected plants that self-pollinated (not visited by bumblebees or on un-touched plants) developed into pods that produced significantly fewer seeds when compared to those from mock-inoculated plants (Figure 6.6). This implies that bumblebee pollination of flowers from CMV-infected plants compensates for seed production loss by restoring the seed numbers to almost the same number as produced by mock-inoculated plants.

6.2.3 Outdoor experiments in the University of Cambridge Botanic Garden: wild bees preferred to visit CMV-infected plants and no preference for BCMV-infected plants.

Initial attempts to assess the response of wild bees to virus-infected plants in the summer of 2016 were unsuccessful due to unforeseen frost. In mitigation, an earlier planting date was chosen for subsequent experiments. The second trial was in the summer of 2017, where I raised cv. Wairimu in the glasshouse in pots and inoculated half the batch with BCMV (Section 2.9) and the other half batch was mock-inoculated. Another batch was raised in the garden, and no inoculation treatment was done to these plants (Figure 6.7A). Upon flowering, glasshouse plants were translocated to the garden and together with ground plants, half the batch was covered with insect netting, the other half remained uncovered (Section 2.9). Aphids settled on stems and leaves of plants that were growing in the ground and a sudden increased infestation by aphids was observed at the onset of flowering. A few days later, I observed the interaction of aphids, ants and ladybirds. Ants were aphid farming and protecting them from predatory ladybirds (Figure 6.7B).

I did initial dawn to dusk observations of pollinators to determine if there was a peak period for

pollinators when floral buds opened up. I observed only one species of bumblebees, *B. pascuorum* (common carder bee) (Figure 6.7D), and it had no peak foraging period (Table 6.1). Over the next seven days between 10:00 am and 14:00 pm, I observed and recorded the frequency of visits of these bees to flowers of both mock-inoculated and BCMV-infected plants. The frequency of visitation was very low, not more than eight visitations per day. Floral visitation frequency was similar to flowers of mock-inoculated plants and BCMV-infected plants (Figure 6.9A). Plants that were grown straight into the ground in the garden were also pollinated by the common carder bee. At some stage, common pollen beetles (*Brassicogethes aeneus* Fibricius) settled on flowers, but they did not cause any floral damage (Figure 6.7B).

Seed yield from mock-inoculated potted plants was significantly higher in covered plants that were not pollinated by common carder bee (Figure 6.8B). There was no significant change in seed yield in BCMV-infected plants that were pollinated by bees and BCMNV-infected plants that were not visited by bees. Notably, seed production was significantly reduced in BCMV-infected plants when compared to that of mock-inoculated plants that were not visited by bees. In plants visited by bees, seed production was similar in BCMV-infected plants and mock-inoculated plants. The lesson I learnt from these results was that shading plays an important role in improving seed yield in cv. Wairimu. This led to the modification of my experimental design on the use of insect netting to include or exclude pollinators (Section 2.9).

In the summer of 2018 (mid-May to mid-September), I conducted my final sets of experiments at the University of Cambridge Botanic Garden with mock-inoculated and CMV-infected cv. Dubbele witte bean market class A and market class B. I failed to complete experiments with mock-inoculated and BCMV-infected cv. Wairimu because they were damaged by muntjac deer at the flowering stage. I raised and inoculated seedlings of Dubbele witte market class A in permanent pots and market class B in temporary pots in the glasshouse (Section 2.9). Dubbele witte market

Table 6.1 Record of bee-visitations on cv. Wairimu from dawn to dusk

Time	Bee species
5 - 6 am	No bees
6 – 7 am	No bees
7 – 8 am	4 X Common carder bees
8 - 9 am	3 X common carder bees
9 - 10 am	3 X common carder bees
10 - 11 am	4 X common carder bees
11 - 12 am	4 X common carder bees
12 - 1 pm	3 X common carder bees
1 – 2 pm	4 X common carder bees
2 – 3 pm	3 X common carder bees
3 – 4 pm	3 X common carder bee
4 – 5 pm	4 X common carder bee
5 – 6 pm	2 X common carder bees
6 – 7 pm	No bees
7 – 8 pm	No bees

class B seedlings were then transplanted into the garden, and they were covered with an aphid-proof mesh until the onset of flowering. At this stage, half the batch of plants was opened on the sides to allow pollinators to forage on flowers and the other half remained closed off on all sides to exclude pollinators (Figure 2.8B). This was done to control for the effect of shading on seed yield as observed in 2017 experiments. I set up a colony of bumblebees (*B. terrestris*) at the centre of plants that were covered by insect netting on top. For ten days between 10:00 and 14:00 hours, I sat in the garden and observed the interaction of bean flowers and their visitors (Figure 2.8C). This involved identifying the pollinators and their pollination behaviour, either from ambient populations or from colony set up in the vicinity (Section 2.9).

In the summer of 2018, I recorded three species of bees that visited cv. Dubbele witte flowers and these were *B. terrestris* (buff-tailed bumblebee) (Figure 6.9A), *B. pascuorum* (common carder bee) (Figure 6.9B) and *Apis mellifera* Linnaeus (European honey bee) (Figure 6.9C). Only the common carder bee was a legitimate pollinator of cv. Dubbele witte flowers because it collected nectar via the corolla mouth. Buff-tailed bumblebees were primary nectar robbers; they would chew a hole at the back of the flower through the corolla and rob the nectar from the hole. Honey bees were secondary nectar robbers that capitalized on holes made by bumblebees to collect nectar.

I went on further to record the flower visitation frequencies of common carder bees on flowers of mock-inoculated and virus-infected plants. Nectar robbing bumblebees recorded very low frequent visits on the flowers (data not shown). The frequency was as up to five visiting bumblebees per day from natural populations. None of the naïve bumblebees from the colony I set up in the plot visited the bean flowers. The common carder bees visited flowers of CMV- infected Dubbele witte market class B plants more frequently than flowers of mock-inoculated plants (Figure 6.10).

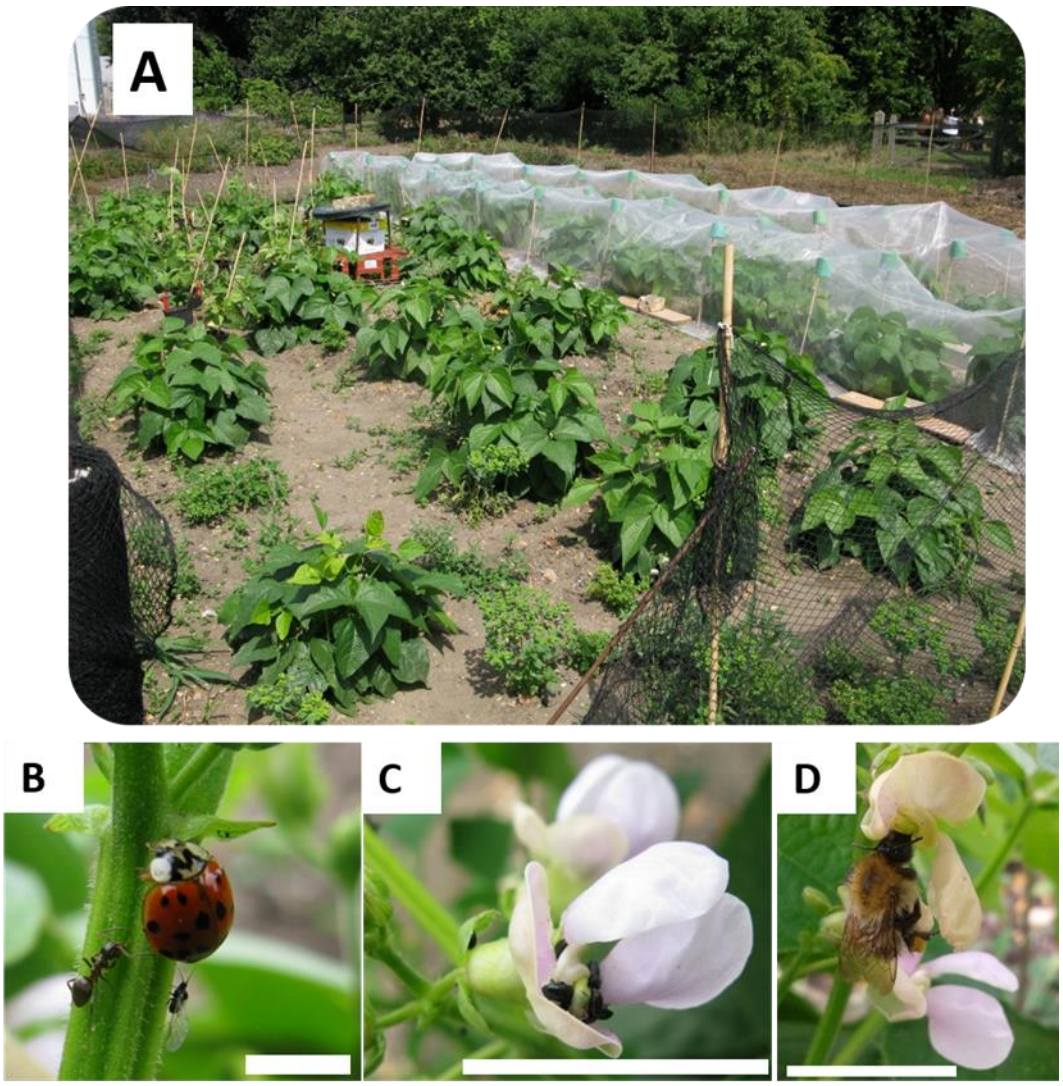


Figure 6.7 Botanic garden trial experimental setup and observations in the summer of 2017. On cv. Wairimu plants that were not covered by insect mash (A), I observed ants farming aphis and protecting them from predatory ladybirds (B). At some stage, pollen beetles settled on flowers for a number of days (C). The common carder bee was the only legitimate pollinator of cv. Wairimu flowers (D). Scale bars (B) = 1.5 cm; (C) = 2.6 cm; (D) = 2.3 cm.

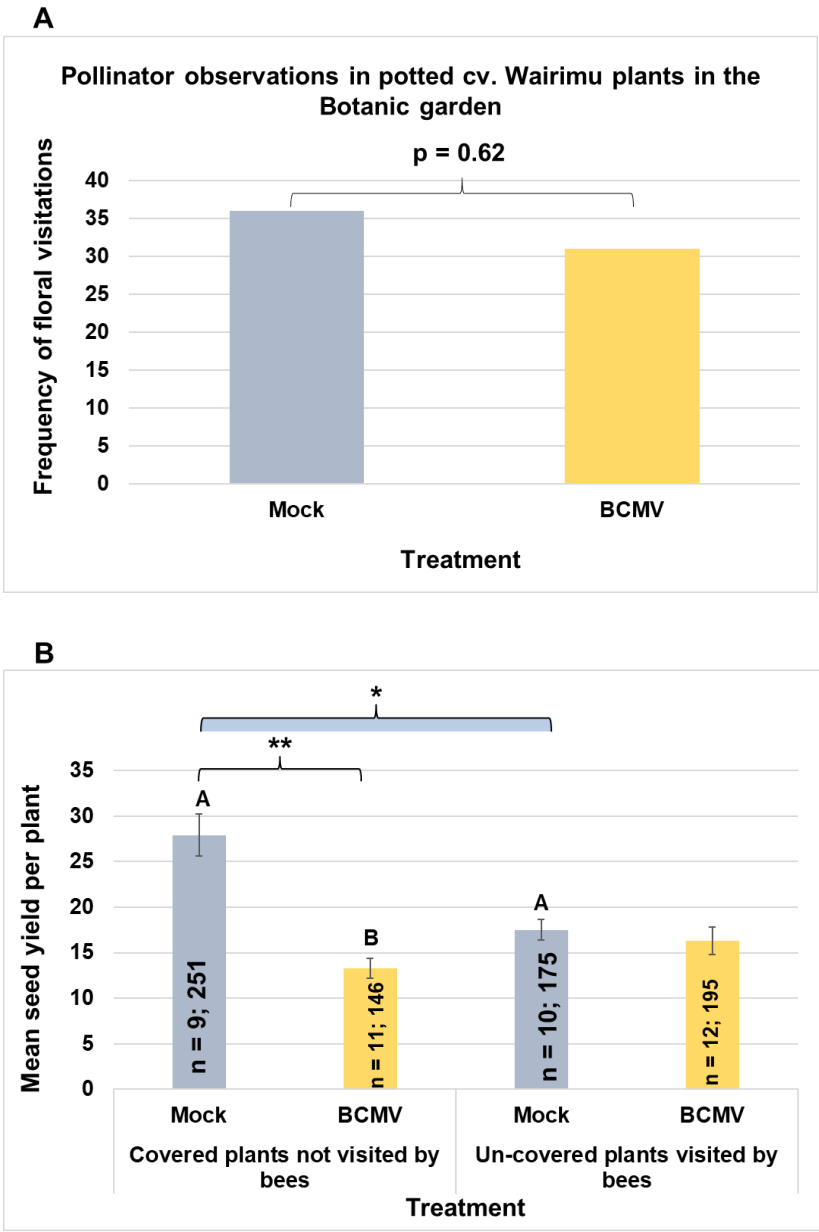


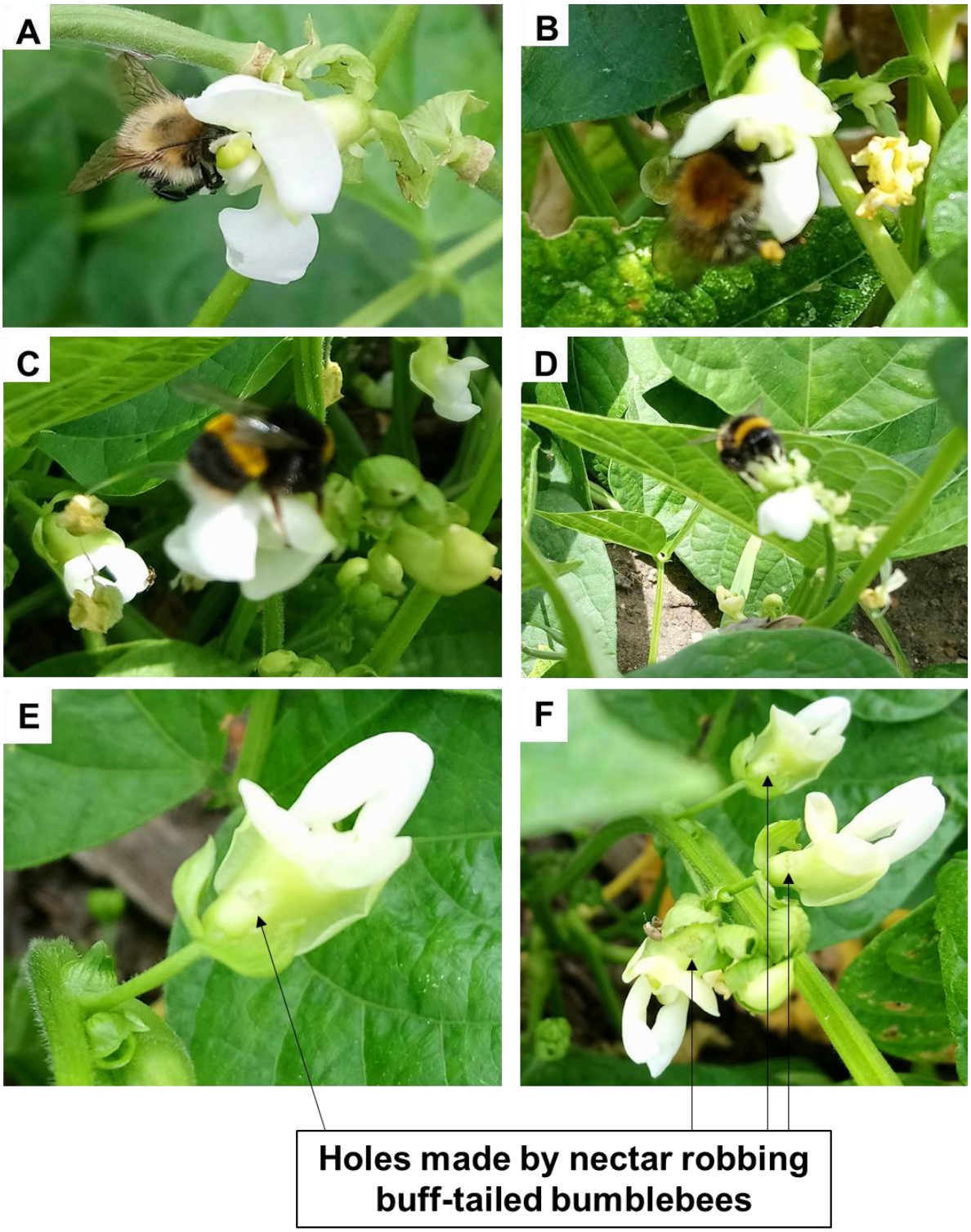
Figure 6.8 Bee-visitation frequency and seed yield results in potted cv. Wairimu plants grown in the botanic garden in the summer of 2017. (A) There was no significant difference in the frequency of floral visitations by common carder bees in mock-inoculated and BCMV-infected plants. (B) Mock-inoculated plants that were covered to exclude bee-visitations on flowers produced the highest seed numbers among all treatments. When compared to BCMV-infected plants that were also not visited by bees, there was a significant difference in seed production (two-sample t-test: p = 0.0001; t = 6.037; df = 18). A marked increase was observed in BCMV-

infected plants that were visited by bees in comparison to BCMV-infected plants that were not visited by bees. Mock-inoculated plants that were not visited by bees produced significantly higher seed yield than mock-inoculated plants that were visited by bees (two-sample t-test; $p = 0.0006$; $t = 4.1762$; $df = 17$). Histogram bar labelling: n = number of plants; number of seeds. Error bars are standard errors around the mean number of seeds.

6.2.4 Bee pollination rescues seed yield in CMV-infected cv. Dubbele witte market class A plants.

When my plants ceased to produce flowers, I removed insect netting from all groups of plants and allowed developing pods to grow to maturity. Pods were harvested and categorized into two groups; (i) pods from flowers visited by bees, and (ii) pods from flowers not visited by bees. This categorization was done in both mock-inoculated and CMV-infected plants. In Dubbele witte market class B plants that were grown in the ground, no seed rescuing was observed in CMV-infected plants that were pollinated by bees (Figure 6.11). Instead, the results showed that bee pollination is beneficial to seed yield, there were no significant differences in seed yield between mock-inoculated plants and CMV-infected plants both in plants not visited by bees and plants visited by bees (Figure 6.11). The differences were noted between plants not visited by bees and plants visited by bees of the same treatment (Figure 6.11). In plants not visited by bees, CMV-infected plants yielded seeds that were almost similar to mock-inoculated plants (Figure 6.11). I would always observe and record seed yield reduction in CMV-infected common bean plants in comparison to mock-inoculated and CMV-infected plants under controlled growth room (Section 2.2.1.2, Chapter 2) and glasshouse conditions.

In potted back-up cv. Dubbele witte market class A plants, there was no significant difference in pod production between mock-inoculated and CMV-infected plants when they were not visited by bees or visited by bees. I observed a significant increase in seed yield in plants visited by bees in both mock-inoculated and CMV-infected plants (Figure 6.12B). Again, seed rescuing was observed in CMV-infected plants, whereby seed production was significantly improved in plants visited by bees (Figure 6.12B). In plants not visited by bees, seed yield in CMV-infected plants was significantly lower than in mock-inoculated plants, but in plants visited by bees, there was no significant difference in seed production between mock-inoculated and CMV-infected plants (Figure 6.12B).


A
B
C
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Holes made by nectar robbing buff-tailed bumblebees

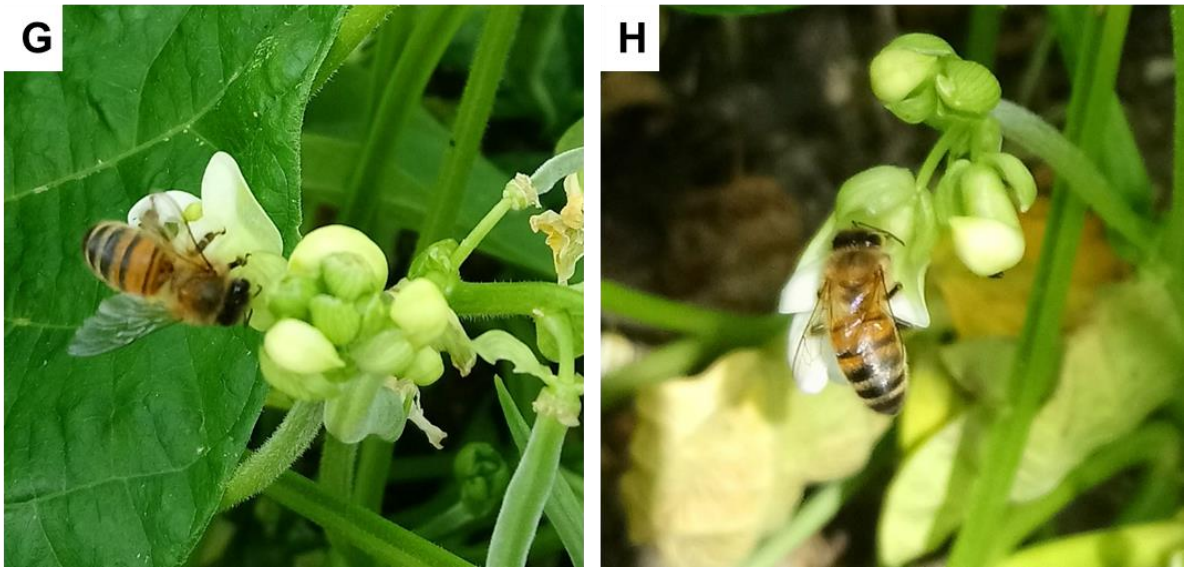

Figure 6.9 Bee visitors of Dubbele witte market class B at the University of Cambridge Botanic Garden, 2018. (A & B) Common carder bee (*Bombus pascuorum*) trip-pollinating fully open flowers. These were the only legitimate pollinators of Dubbele witte in the Botanic garden. (C & D) Buff-tailed bumblebees drilling holes and robbing nectar from fully open flowers. (E & F) Holes on the corolla tube made by nectar-robbing bumblebees. (G & H) Honey bees capitalizing on holes made by bumblebees, collecting nectar from the holes.

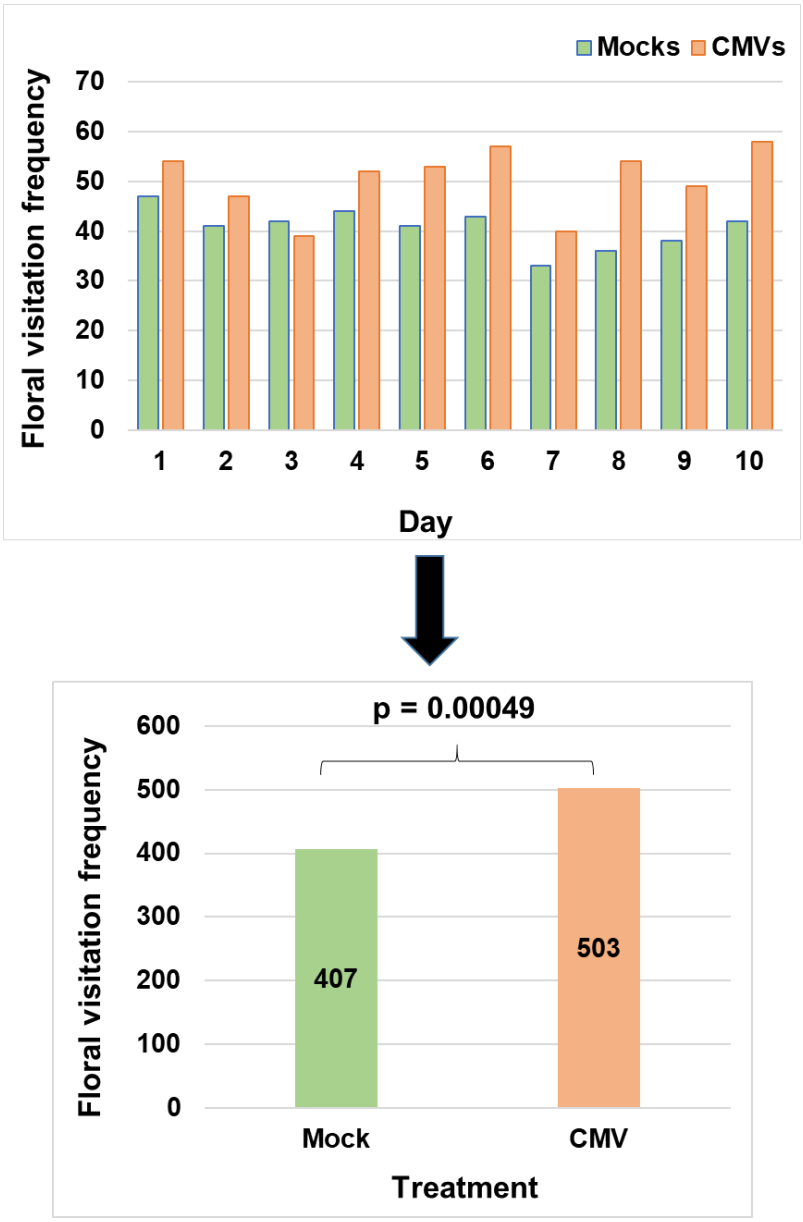

Figure 6.10 Frequency of visitations on flowers of Dubbele witte market class B by the common carder bees in the University of Cambridge Botanic Garden over 10 days in the summer of 2018. The daily and pooled floral visitation frequencies of the common carder bees on flowers of mock-inoculated and CMV-infected plants recorded in 10 days is shown. Common carder bees made more significant frequent visits to flowers on CMV-infected plants than they did on mock-inoculated plants.

I speculated that the shading effect of the aphid-proof mesh and insect netting might have conditioned CMV-infected Dubbele witte market class B plants that were raised in the garden to perform better than mock-inoculated plants as they were growing under an aphid-proof mesh. Hence, I tested the effect of shading on seed yield under glasshouse conditions. Half a batch of cv. Dubbele witte market class B plants were grown covered with aphid-proof mesh, and the other half was grown un-covered. Upon flowering, the aphid-proof mesh cover was replaced with insect-netting, and after flowering, the net was removed. Pods were harvested, and seed yield was counted. I had problems with spider mites that were controlled by bio-controls, and they might have possibly interfered with the growth of my experimental plants. Hence the results I got might be inconclusive. I observed a reduction in pod production in mock-inoculated plants that were covered by insect netting (Figure 6.13). When pod production of mock-inoculated and CMV-infected plants of covered plants treatment was compared; there was a marked difference, as observed in the botanic garden plants (Figure 6.12).

6.2.5 Bee-pollination reduces the rate of viral seed-transmission

I used cv. Wairimu for rate of seed-transmission of viruses experiments in BCMV, BCMNV and CMV-infected parent plants. Plants that were grown in the controlled growth room (Section 2.2.1.2, Chapter 2) were used for experiments with BCMV, BCMNV and CMV and those that were grown in the glasshouse and Botanic garden were used in experiments with BCMV only. Seeds that were produced by virus-infected plants were germinated, and radicles from developing seedlings were collected for screening for presence of viruses using ELISA (Section 2.4.5, Figure 2.2; Chapter 2). The rate of seed-transmission in progeny of plants not visited by bees grown under controlled growth conditions (Section 2.2.1.2, Chapter 2) was lowest in CMV-infected parent plants, with only just an 8% rate (Table 6.2). This was followed by progeny of BCMV-infected parent plants with a 16% rate, and the highest was recorded in progeny of BCMNV-infected parent plants with a transmission rate of 33% (Table 6.2). Under glasshouse conditions, BCMV-infected

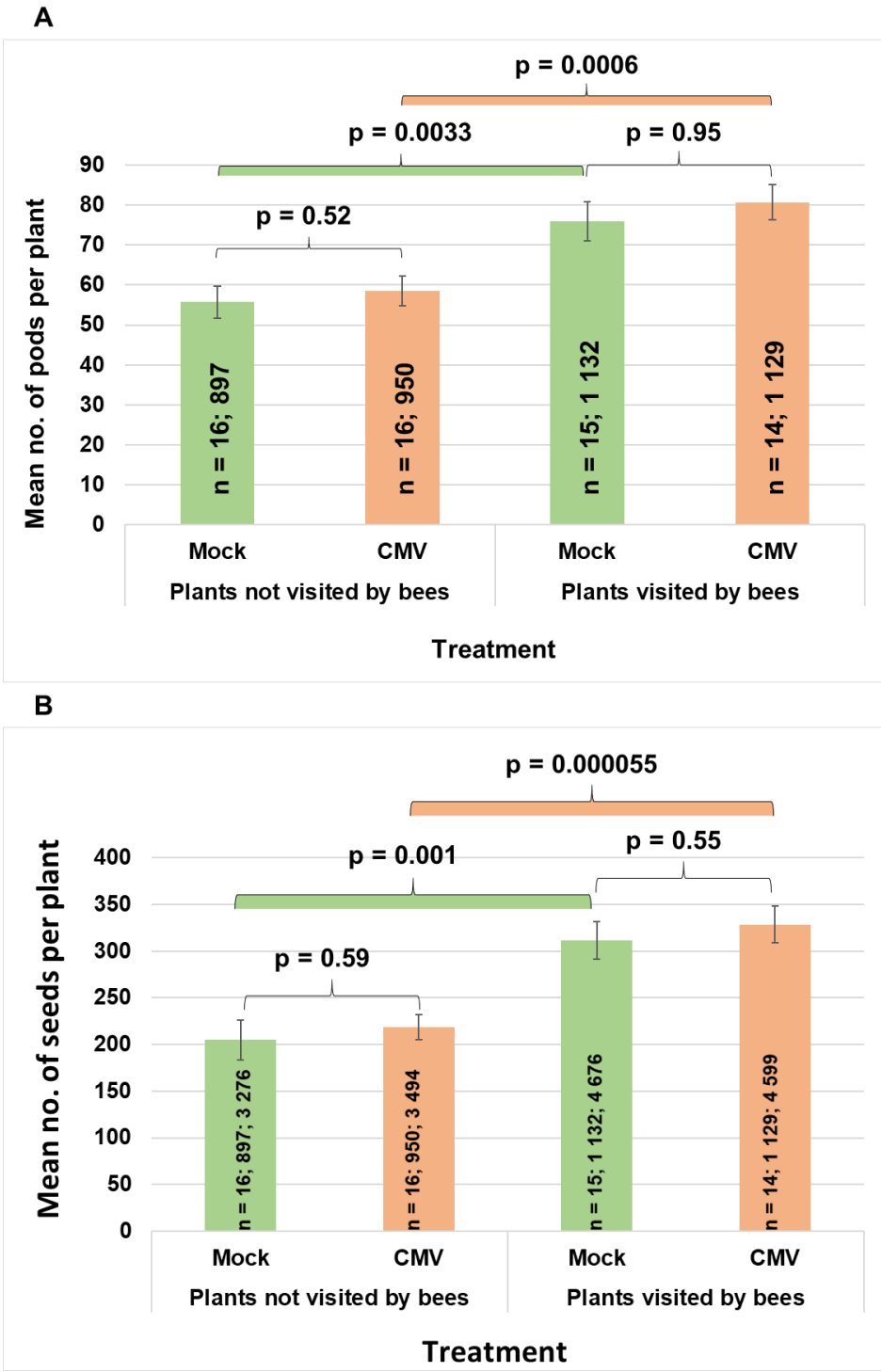

Figure 6.11 Pod and seed production in Dubbele witte market class B at the University of Cambridge Botanic Garden, 2018. Pollination by the common carder bee increased (A) pod production and (B) seed production per plant. (B) Notably, bee-visitation increased seed yield by over 30% in both mock-inoculated (two-sample t-test t = 3.66; df = 29) and CMV-infected plants

(two-sample t-test t = 4.75; df = 28). The p-values are from unpaired two-sample t-tests. Histogram bar labelling: n = number of plants; number of pods; number of seeds. Error bars are standard errors around the mean number of seeds.

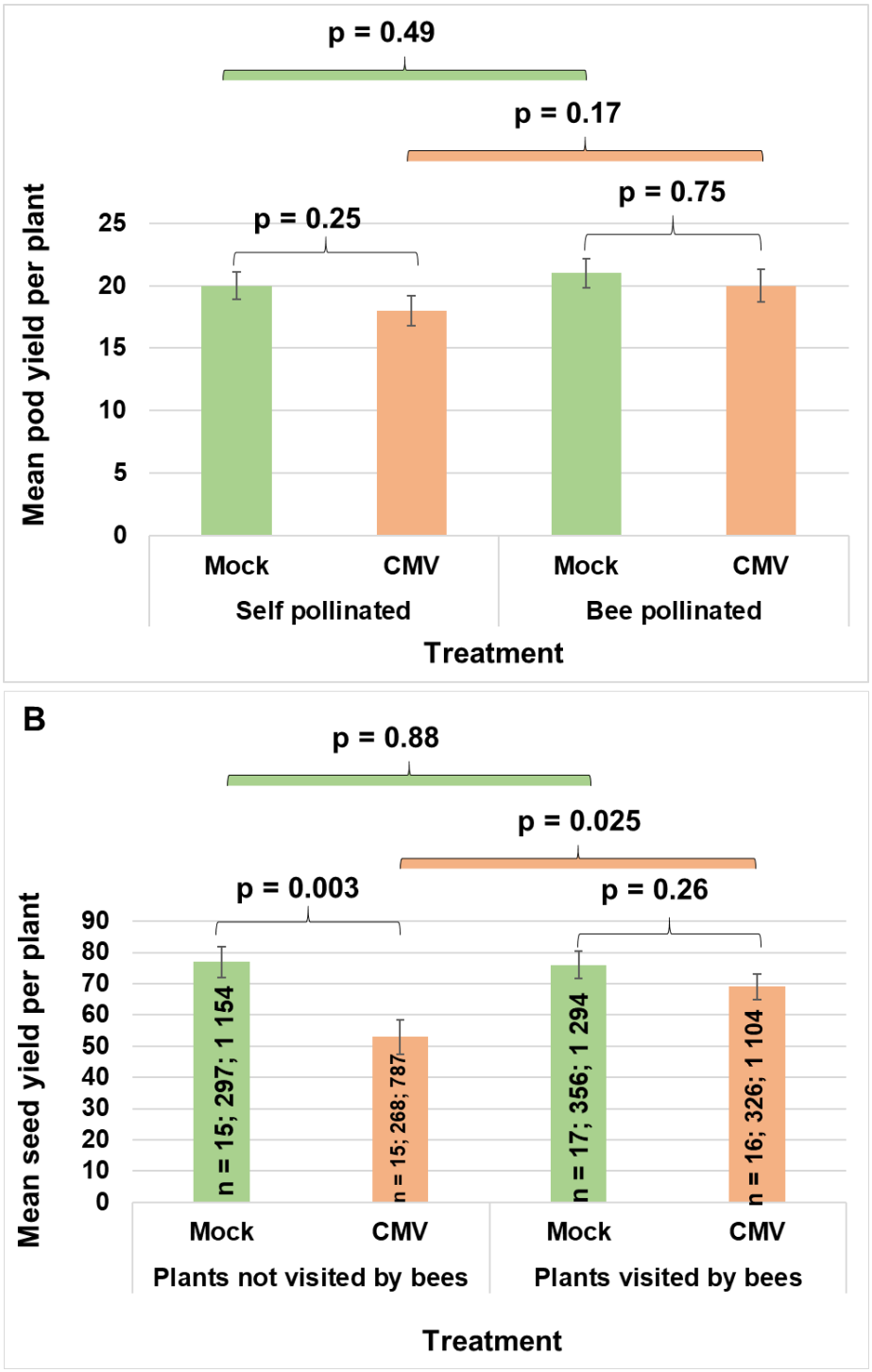

Figure 6.12 Seed production in potted Dubbele witte market class A in the University of Cambridge Botanic Garden, 2018. These plants were raised in the glasshouse and translocated to the botanic garden at the onset of flowering. Half the batch of both mock-inoculated and CMV-infected plants was covered on top and all sides to prevent pollinators from accessing the plants. The other half batch was only covered on top to allow pollinators to visit. (A) In both plants that

were not visited by bees and plants visited by bees, pod production was not significantly different in mock-inoculated and CMV-infected plants. (B)Where pollinators were excluded, CMV infection caused a significant reduction in seed production (two-sample t-test; t = 3.26; df = 28). When pollinators had access to flowers, CMV-infected plants significantly improved in seed production in comparison to CMV-infected plants that were not visited by bees (two-sample t-test; t = 2.36; df = 29). The p-values are from unpaired two-sample t-tests. Histogram bar labeling: n = number of plants; number of pods; number of seeds. Error bars are standard errors around the mean number of seeds.

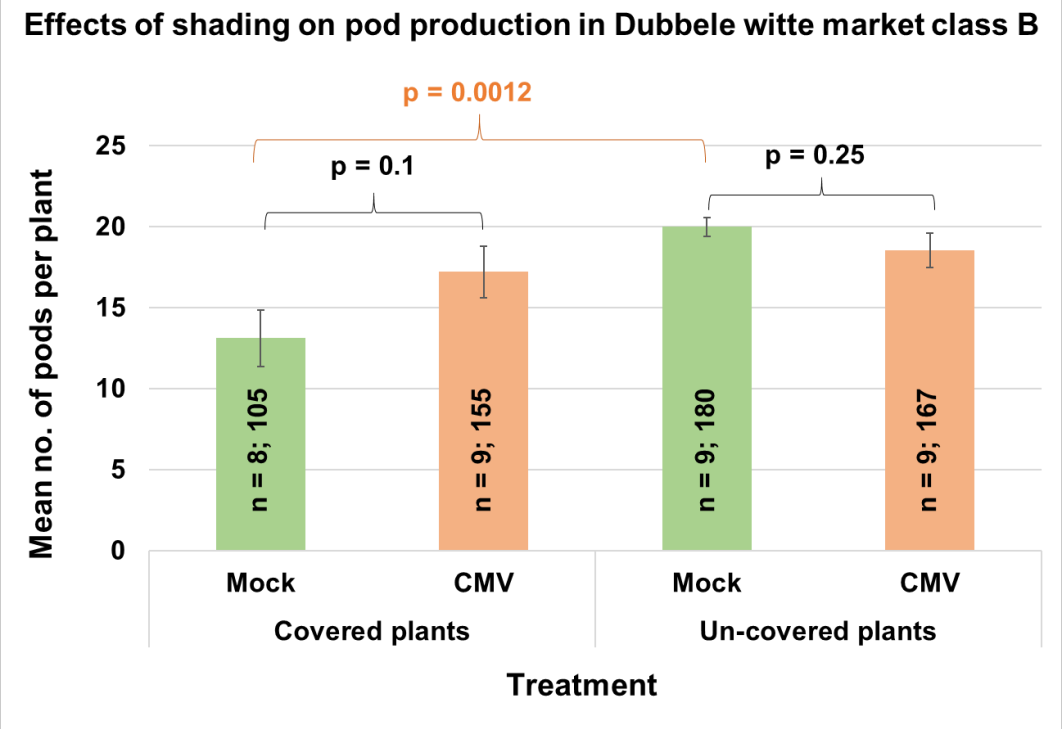

Figure 6.13 Shading effect of aphid-proof mesh and insect netting on Dubbele witte market class B in the glasshouse in 2019. The results suggest that shading provided by aphid-proof mesh and insect netting reduced pod production in mock-inoculated plants. However, these findings might not be reflective of the entire truth because the plants were infested with spider mites. The p-values shown are from unpaired two-sample t-tests. Histogram bar labelling: n = number of plants; number of pods. Error bars are standard errors around the mean number of pods.

Table 6.2 Rate of seed-transmission of BCMV, BCMNV and CMV in cv. Wairimu

***Self-pollinated plants grown under controlled growth room conditions**					
Virus	No. of parent plants that produced tested progeny	No. of parent plants that produced virus-infected progeny	No. of progeny seedlings tested	No. of progeny that tested positive	Rate of seed-transmission
BCMV	18	8	109	20	18%
BCMNV	24	15	102	35	34%
CMV	24	5	103	11	11%
***Self-pollinated plants grown under glasshouse conditions**					
Virus	No. of parent plants that produced tested progeny	No. of parent plants that produced virus-infected progeny	No. of progeny seedlings tested	No. of progeny that tested positive	Rate of seed-transmission
BCMV	10	6	121	21	17%
Covered plants not visited by common carder bees in the University of Cambridge Botanic Garden					
Virus	No. of parent plants that produced tested progeny	No. of parent plants that produced virus-infected progeny	No. of progeny seedlings tested	No. of progeny that tested positive	Rate of seed-transmission
BCMV	10	8	122	28	20%
Plants pollinated by common carder bees in the University of Cambridge Botanic Garden					
Virus	No. of parent plants that produced tested progeny	No. of parent plants that produced virus-infected progeny	No. of progeny seedlings tested	No. of progeny that tested positive	Rate of seed-transmission
BCMV	10	3	148	11	7%

Note: in this table, ***** "self-pollinated" indicates no contact with bees.

Table 6.3 Summary table of results from pollination experiments in the glasshouse and University of Cambridge Botanic Garden

Treatment	Mock	BCMV	CMV
Glasshouse bumblebee free foraging experiments			
Dubbele witte market class A	Fewer visits on flowers by bumblebees	N/A	More significant frequent visits on flowers by bumblebees
Wairimu	Same frequent visits on flowers as BCMV-infected plants. More significant visits on fowers than CMV-infected plants	Same frequent visits on flowers as mock-inoculated plants.	Less frequent visits on flowers as compared to mock-inoculated plants.
Nectar volume and sucrose concentration in cv. Dubbele witte market class A			
In controlled conditions (PGF)		N/A	Significantly increased compared to mock-inoculated plants flowers
In glasshouse conditions		N/A	Significantly increased compared to mock-inoculated plants flowers
Seed production in plants used in pollination experiments in glasshouse conditions			
cv. Dubbele witte market class A	Seed production significantly	N/A	Significant increase in seed yield in plants

	increased in plants visited by bees. No seed rescuing.		visited by bees and no significant difference when compared to mock-inoculated plants visited by bees. Seed production restored to match that of mock-inoculated plants not visited by bees. Thus, seed rescuing
cv. Wairimu	Seed production significantly increased in plants visited by bees. No seed rescuing.	No results obtained, plants were damaged by pests and high temperatures on separate episodes.	Significant increase in seed yield in plants visited by bees and no significant difference when compared to mock-inoculated plants visited by bees. Seed production restored to match that of mock-inoculated plants not visited by bees. Thus seed rescuing
Pollination experiments in the University of Cambridge Botanic garden			

cv. Dubbele witte market class B	Less frequent visits by common carder bees.	N/A	More significant frequent visits by common carder bee than mock-inoculated plants.
cv. Wairimu	Same frequent visits by common carder bee as BCMV-infected plants.	Same frequent visits by common carder bee as mock-inoculated plants.	Plants damaged by muntjac deer.
Seed production in plants used in pollination experiments in glasshouse conditions			
Potted cv. Dubbele witte market class A plants	Significant increase in seed yield in plants visited by bees.	N/A	Significant increase in seed yield in plants visited by bees and no significant difference when compared to mock-inoculated plants visited by bees. Seed production restored to match that of mock-inoculated plants not visited by bees. Thus seed rescuing
cv. Dubbele witte market class B	Significant increase in seed yield in plants visited by bees.		Significant increase in seed yield in plants

			visited by bees. No seed rescuing.
Potted cv. Wairimu	Significant increase in seed yield in covered plants (shading effect provided favourable conditions)	No change in seed yield in plants visited by bees.	N/A

parents that were not visited by bees produced progeny with 17% BCMV-transmission rate (Table 6.1). Progeny from parent plants that were covered and not visited by common carder bees in the Botanic garden had a seed-transmission rate of 20% (Table 2.1). Pollination by common carder bees in the Botanic garden elicited a reduction in BCMV seed-transmission rate to 7% (Table 2.1). I randomly tested the presence of viruses in seed cotyledons, testae and radicles from the same seed and I observed that some cotyledons would be infected, but the radicle would be virus free. All virus-infected radicles were associated with virus-infected cotyledons. None of the tested seed testae were virus-infected (data not shown).

6.3 DISCUSSION

In this chapter, I have shown that CMV-infection induced an increase in nectar production and nectar sucrose concentration under glasshouse conditions in Dubbele witte market class A but did not induce any changes in controlled growth conditions (Table 6.3). Bumblebees in the glasshouse exhibited what I concluded to be associative learning pollination behaviour towards more rewarding flowers (Table 6.3). Thus, naïve bees increasingly visited more rewarding flowers, guided by visual, olfactory and tactile cues. When buff-tailed bumblebees and common carder bees pollinated flowers of both mock-inoculated and CMV-infected common bean plants, a significant increase was observed when compared to seed yield from plants that were not visited by bees. Interestingly, seed yield in CMV-infected plants was increased by bee-pollination to the same level as mock-inoculated plants, hence seed yield compensation. This tremendous seed yield recovery in CMV-infected plants that were visited by bees supports the hypothesis that viruses repay their susceptible hosts by attracting pollinators to ensure reproduction success thereby passaging alleles of virus susceptibility in their offsprings with possible long-term consequences for virus-host co-evolution as initially proposed by Groen *et al*. (2016).

The common carder bee was the only legitimate pollinator of common bean in the botanic garden. The buff-tailed bumblebees were primary nectar robbers, and European honey bees were secondary nectar robbers. These results demonstrate that foraging behaviour of bees is species-specific. Despite being less frequent visitors of Dubbele witte plants, the common carder bee proved to be an effective and specialised pollinator that resulted in increased yield of all pollinated plants in comparison with pollinator exclusion treatments. Marzinzig and colleagues (2018) reported similar observations in *Vicia faba* (faba bean) whereby the two locally dominant pollinator species, *Apis mellifera* and *B. terrestris/B. lucorum* mostly robbed nectar from flowers. Instead, the less frequent pollinator species, *B. hortorum* L., revealed to be the most efficient pollinator that increased seed yield in plants that were visited by bees as compared to plants where bee pollinators were excluded (Marzinzig *et al*. 2018). In experiments with BCMV infection in the botanic garden, covered cv. Wairimu plants were an exceptional case because they produced more seeds than plants visited by bees, most probably because of the effect of shading.

My results have shown that bees prefer to pollinate flowers that offer better rewards through associative learning. Mock-inoculated cv. Wairimu and CMV-infected cv. Dubbele witte market plants were increasingly preferred by foraging bees because they offered better nectar rewards. My findings are well supported in literature, whereby it has been shown that bumblebees can learn effectively to associate particular floral features with nectar and pollen rewards (Cnaani *et al*. 2006; Raine *et al*. 2006; Gomez *et al.* 2008; Eisenhardt 2014; Konzmann & Lunau 2014). In Chapter 4, I showed how viruses induce changes in plant emitted VOCs in common bean. It is possible that foraging bumblebees were guided mostly by differences in VOCs to distinguish and establish which group offered better reward between mock-inoculated and virus-infected plants.

Virus seed transmission is achieved either directly through invasion of the embryo via the ovule or indirectly through invasion of the embryo, mediated by infected gametes. In direct embryo

invasion, Wang and Maule (1994) showed that viruses invade the embryo via the suspensor, which functions as a conduit for nutrient flow to support growth of the embryo. Both processes operate simultaneously for some viruses in certain hosts as observed in barley stripe mosaic virus in barley (Mandahar 1981). I noted that the presence of virus in the seed does not always lead to seedling infection. That is, BCMV, BCMNV and CMV may be present in all parts of the seed, including embryo, and cotyledons, but still might not be transmitted parentally if it does not reach the embryo during shoot growth during germination. Hence, I conclude that embryo infection is likely via the suspensor, both from parent to offspring and from seed to developing seedling. I propose that the rate of seed transmission of these three viruses depend on how fast they move across the suspensor before it degenerates as well as the genetic makeup of the developing seedling in cv. Wairimu hosts.

The reduction in seed-transmission rate of BCMV in cv. Wairimu plants that were visited by bees when compared to plants where bee-pollinators were excluded suggest that the genetic complement of the maternal or progeny tissues determines the efficiency of seed transmission. Plants that self-pollinate produce progeny with less genetic diversity, since genetic material from the same plant is used to form gametes, and eventually, the zygote (Raijmann *et al*. 1994). In contrast, parents that were visited by bees produced progeny with greater genetic diversity because the microgametophyte and megagametophyte are derived from different plants Raijmann *et al*. 1994. Thus, progeny from BCMV-infected plants not visited by bees were highly susceptible to BCMV in the seed than cross-pollinated (in this case plants visited by bees) progeny. My hypothesis still stands, whereby bee-pollination ensures the spread of the alleles of virus-susceptibility in progeny, although the rate of vertical seed-transmission is lowered. In Section 3.3.1 of Chapter 3, I explained how synchronised flowering of virus-infected and healthy plants promote gene flow. The same explanation is applicable in this case whereby alleles of virus susceptibility are introduced in progeny from healthy plants and also alleles of resistance are

introduced into progeny of virus-infected plants by pollinators moving pollen around. Vertical seed-transmission is known to reduce virus accumulation and virulence. Thus plant-virus co-evolution is promoted (Pagán *et al*. 2014).

My work also supports the idea of Lederberg (2000), who suggested that microbes might have evolved in ways that ensure survival of susceptible hosts since if they did not, they would cause their own extinction. In this chapter, I have shown that CMV infection induced the production of more rewarding nectar in cv. Dubbele witte market class A, and bumblebees increasingly visited these plants. This resulted in seed production rescuing in CMV-infected plants. There are other underlying mechanisms we are yet to understand, that explains how bee pollination in CMV-infected cv. Wairimu elicited seed rescuing even though they received significantly lower pollination services from bumblebees as compared to mock-inoculated plants (Figure 6.2). In Chapter 4, I showed how viruses alter emitted VOCs in common bean. I propose that the associative learning pollination behaviour by bumblebees as they visited more rewarding plants in the glasshouse is mainly driven by the quality of reward and reinforced by VOCs cues at large.

This study is novel in investigating the interaction of plant viral pathogens and bee pollination and their combined effects on reproduction success. My findings provide robust evidence that pollination services can elicit partial yield compensation in the presence of plant viral pathogens in both controlled glasshouse conditions and pristine environments. Yield compensation in virus-infected plants promotes the reproductive success and passaging of alleles conferring virus susceptibility. As suggested by the mathematical model in Groen *et al*. (2016), this could result in virus susceptible plants persisting in natural landscapes. The results also provide evidence that pollination services increase seed yield in partially pollinator-dependent and self-compatible crops.

CHAPTER 7

GENERAL DISCUSSION

7.1 Effects of plant viral pathogens on plant-pollinator relationships

The effects of virus infection on pollinators and the effects of virus-induced alterations of bee-plant interactions on seed yield had not been studied until the novel studies by Groen and colleagues (2016). They proposed the 'payback' hypothesis which states that viruses might pay back their susceptible hosts by making them more attractive to pollinators, thereby promoting reproduction and persistence of virus susceptible alleles in nature (Groen *et al*. 2016). My work was an extension and investigation of the payback hypothesis using a different host, a different pollination syndrome, and two viruses not used in the previous study (Groen *et al*. 2016). In addition to controlled environment experiments (Section 2.2.1.2, Chapter 2), I also conducted experiments in the glasshouse with commercial bees and 'field' environments at the University of Cambridge Botanic Garden with feral bees.

My results with respect to testing the payback hypothesis had the following key findings: (i) the payback hypothesis is tenable in different plant hosts; (ii) VOC mediated differences on bee behaviour in plants exhibiting different pollination syndromes are induced by more than one virus; (iii) virus-infected plants appear to emit 'honest' signals since pollinators receive a richer nectar reward, but under certain conditions and these signals are host-specific; (iv) viruses pay back common bean plants by improving their reproductive fitness in glasshouse conditions and field conditions, (v) bee-pollinators benefit from pollinating virus-infected plants by receiving high-quality nectar reward under certain conditions and in different hosts, and (vi) virulence determines whether the virus pays back its host or not. These results suggest that virus 'pay back' their hosts, and this is mediated through altering VOC blends to attract pollinators and enhancing nectar reward quality and quantity. The resulting enhancement of bee pollination services results in

recovery of seed production in virus-infected plants and thus increased host plant reproductive fitness.

Virulence of viruses used in this study played an important role in determining whether the particular virus would payback it susceptible host or not and how the susceptible hosts would respond to the level of virulence. BCMNV is a virulent virus, and indeed, I observed severe symptoms it caused in common bean, infected plants were more stunted, and leaves were severely deformed (Figure 3.1, Chapter 3). BCMNV-infected plants flowered much later than all other treatment groups. According to Robert-Seilaniantz *et al*. (2007), GA seems to facilitate defence against viruses by partially modulating SA and JA/Et-mediated signalling pathways. I speculate that as a counter defence, BCMNV viral proteins may interfere with the GA signalling pathway to a greater extent and hence causes severe stunting and prolonged delay of flowering than with BCMV. Other changes observed in BCMV and CMV-infected plants were not observed in BCMNV-infected plants. These include increased nectar volume and sucrose concentration (Chapter 3), unique VOCs emitted by BCMNV-infected plants (Chapter 4) and emission of VOCs in flowering plants that elicited innate attractiveness to bumblebees (Chapter 5). GA is an important hormone that promotes stem elongation and modulates flowering (Sun & Gubler 2008).

7.2 Bumblebees show an innate preference for VOCs emitted virus-infected plant

Schiestl & Johnson (2013) gathered evidence to support the idea that the relationship between plants and pollinators can be based on either pollinator sensory bias, associative learning or co-evolution. In many such relationships, the class and composition of VOCs in the scents overlap (Waelti *et al*. 2008; Steiner *et al*. 2011). Studies of the biological effects of virus-induced changes in plant VOC profiles on insects are dominated by work on vectors (Mauck *et al*. 2012; Dorokhov & Komarova, 2014). There are few studies on the foraging strategies of bees focusing on innate

olfactory cues (Menzel 1985) or on specific VOCs that are important in innate biases of naïve bees (Ariela *et al*. 2017). Some bees may exhibit a sensory bias towards a specific VOC and disregard honest signals from rewarding flowers as observed by Ariela and colleagues (2017) in bumblebees foraging on seep monkeyflowers (*Mimulus guttatus* Fisch. ex DC.). In their study, Aruela *et al*. (2017) observed that naïve bumblebees had an innate preference for VOC blends with higher quantities of β-*trans*-bergamotene and experienced bees preferred floral VOC blends of non-rewarding outbred lines of seep monkeyflowers over rewarding inbred lines. Outbred lines of seep monkeyflowers emitted larger quantities of β-*trans*-bergamotene as compared to inbred lines (Ariela *et al*. 2017). They suggested that their observations may represent an elusive form of deceit-pollination which is an energy-efficient benefit to plants that minimizes costly production of rewards while still allowing plants to attract pollinators (Ariela *et al*. 2019).

My results in Chapter 4 show that the innate preference of VOC blends from virus-infected plants was not driven by potential honest signal cues because the reward was similar to mock-inoculated plants. When visiting flowers, experienced bees learn to associate floral traits with rewards and hence prefer honest signals that indicate rewarding flowers (Knauer & Schiestl 2015). Instead, the innate preference was driven by sensory biases for VOC blends from virus-infected plants (Chapter 5). Deceptive pollination occurs in orchids that have non-rewarding flowers yet still attract pollinators and the pollination services increase plant reproductive fitness (reviewed in Jersáková *et al*. 2006). Hence further studies are required to validate the specific volatiles from virus-infected plants that are preferred by bees, particularly from non-flowering plants that seem to attract the bees more effectively than flowering plants.

7.2.1 Green leaves are a key source of bee-attracting VOCs

Free-choice assays showed that buff-tailed bumblebees were attracted to VOCs emitted by both flowering and non-flowering BCMV and CMV-infected plants, and non-flowering BCMNV-infected

plants, indicating that leaves were the primary source of attractive volatiles. (Chapter 5). The emission of pollinator-attracting VOCs by green leaves is not unique to common bean. Previous work in our lab found that bumblebees showed similar innate preferences for VOC blends from flowering and non-flowering CMV-infected tomato plants, indicating that leaves a source of attractive volatiles (Groen *et al*. 2016). Studies in the dwarf palm (*Chamaerops humilis* L.) have shown that leaves produce scents that attract pollinators from a long-distance, whereas scents from inflorescence are not attractive from a distance (Dufaÿ *et al*. 2003; Caissard *et al*. 2004). I suggest that green leaf volatiles act in tandem with floral volatiles to attract pollinators from long and short distances.

My findings (Chapter 5) thus offer an opportunity to explore the sensory basis of virus-infected plant-pollinator interactions by determining the minimal subset of green leaf VOCs necessary and sufficient to drive the innate olfactory and behavioural preferences of naïve bumblebees. Future studies might also focus on how viruses alter the specific biochemical pathway from which virus-induced VOCs that are preferred by bees are produced. For example, an in-depth understanding of how viruses interfere with the MEP/DOXP pathways localized to plastids and alter the emission of monoterpenoids, linalool and the linalool oxides (Lichtenthaler *et al*. 1997; Raguso & Pichersky 1999).

7.2.2 Application of virus-induced VOC emissions in agriculture

According to Bailes *et al*. (2015), floral traits such as volatiles are promising and may provide a gateway for crop yields improvement, yet little explored. Plant breeders mainly focus on breeding crops for pest, disease and drought resistance, and in the process, most likely important traits such as pollinator-attracting volatiles are lost (Bailes *et al*. 2015). The qualitative and quantitative changes of volatiles observed in common bean plants and the innate preference for the induced emitted VOCs by bumblebees in Chapters 4 and 5 may guide plant breeders to improve pollinator

services to their crops. Again, future work should assess the attractiveness of VOCs that were increased or decreased in emission rates by virus infection in *P. vulgaris* in different ratios to bumblebees to determine the best combination to breed for. This could help plant breeders and pollination experts ensure that crops achieve optimal pollination service while utilizing minimum resources. It is essential for breeders to look out for and avoid enhancement of volatiles that may attract florivores, rather than pollinators, and cause a decrease in seed production, as observed in the wild Texas gourd, *Cucurbita pepo* var. *texana* (Scheele) D.S.Decker (Theis & Alder 2012). Theis & Adler (2012) experimentally elevated floral emission of 1,4-dimethoxybenzene in *C. pepo* var. *texana*, considered the most attractive compound to specialist squash bee pollinators. They observed decreased plant reproduction as a result of the concomitant attraction of florivorous beetles, rather than desired pollinators. Glasshouse and field experiments are needed to explore the effects of virus infection on bumblebee-meditated pollination and the resulting plant reproductive success. Field studies would also be helpful to determine if the changes in VOC blends induced by virus infection affects the disease and pest resistance of *P. vulgaris*.

Other authors have suggested the use of VOCs in plant systematics and taxonomy because the chemistry of VOCs is species-specific (Dobson 2006; Edris 2007; Dudareva *et al*. 2013; Kumari *et al*. 2014). Jansen *et al*. (2011) reflected on how technological developments in the field of analytical chemistry can be applied in an agricultural setting to detect plant diseases by analysis of VOC emission. Jansen and colleagues (2009) demonstrated the feasibility of detecting plant damage based on plant-emitted VOCs at greenhouse scale. In Chapter 5, I observed that virus-induced emitted VOCs from common bean are specific to virus strain, particularly in BCMNV and CMV-infected plants. I suggest that virus-induced changes in VOC emission have potential for use in detection and characterisation of phytopathogens, especially in highly intensive greenhouse agriculture. Greenhouse based agriculture is very popular, for example, in countries like the Netherlands. Global climate change might also see the increased use of greenhouses for

crop yield security (Jacobs *et al*. 2019), and hence viral disease detection using VOCs will become very useful. There is the need for plant virologists, analytical chemists and engineers to work together and explore the development of an instrument that can be used in agricultural fields to detect viral pathogens. According to Jansen *et al*. (2011), the device should have good accuracy and precision, high sensitivity, a wide dynamic concentration range and favourable combination of high selectivity and resolution and I support their suggestions

7.3 Viruses sometimes induce increased nectar production and increased nectar sucrose concentration in common bean.

Floral morphology and reward influence how likely pollinators will visit them. As shown by several authors, bees prefer to visit flowers that offer greater rewards usually in the form of pollen or nectar (Shafir *et al*. 1999; Cnaani *et al*. 2006; Raine *et al*. 2006; Gomez *et al.* 2008; Konzmann & Lunau 2014; Mallinger & Prasifka 2017). In Chapter 3, I showed that under growth room conditions where light levels are lower than natural daylight (200 µmol/m²/s *versus* an average of about 950 µmol/m²/s, respectively), infection by BCMV and CMV induced an increase in the volume of nectar and nectar sucrose concentration in flowers of cv. Wairimu plants. I also showed that virus infection induces changes in VOCs emitted by flowers of infected plants. In glasshouse conditions, flowers of CMV-infected of cv. Dubbele witte market class A plants produced higher volumes of nectar with more concentrated sucrose in comparison to mock-inoculated plants
(Chapter 6). In a flight arena in the glasshouse, foraging bumblebees preferred to visit more rewarding CMV-infected cv. Dubbele witte market class A plants over mock-inoculated plants. This pollination behaviour suggested that the bumblebees were learning to forage on more rewarding flowers more frequently.

In Chapter 4, I showed how viruses induce changes in VOCs emitted by their hosts and in Chapter 5 that bumblebees have an innate preference for VOC blends emitted by virus-infected plants. Bees cannot be deceived for long by attractive volatiles before they realise that there is no

beneficial reward. Olfactory cues are easily remembered and learned by pollinators and VOCs are often the basis upon which flowers pollinators choose to visit (Goulson *et al*. 2001; Wright & Schiestl 2009). CMV infection in Dubbele witte market class A induced the production of more rewarding nectar to foraging bees; higher nectar quantities and nectar sucrose concentration. Taken together, CMV-infected Dubbele witte plants appeared to emit 'honest' VOC signals since pollinators were receiving a richer nectar reward, under glasshouse conditions and in near-natural conditions in the University of Cambridge Botanic Garden. I also speculate that the emission of honest signals under low light conditions whereby viruses induce the production of better-quality nectar rewards will benefit virus-infected plants through attraction of pollinators. The ability of viruses to induce increased nectar production and nectar sucrose concentration is, however, not universal to all bean varieties and virus species. My results have shown that these attributes are host genotype-specific, virus species-specific as well as dependent upon growing conditions or environment.

On the other hand, dishonest signals from flowering cv. Wairimu infected with CMV or BCMV under glasshouse conditions did not attract more frequent visits by bumblebees. Both BCMV-infected plants and mock-inoculated plants were visited equally by bumblebees because they offered equal rewards (Chapters 3 & 6), although VOC blends they emitted were attractive to bumblebees (Chapter 5). CMV-infected cv. Wairimu plants received less frequent visits by bumblebees (Chapter 6), regardless of their volatiles innately attracting bumblebees (Chapter 5). Taken together, dishonest signals do not pay back BCMV and CMV-infected cv. Wairimu plants under glasshouse conditions and BCMV-infected plants under garden conditions because they are overshadowed by reward quality.

There is a possibility that increased nectar production and nectar sucrose concentration may be a plant response mechanism in the presence of viral pathogens to enhance reproduction by

attracting pollinators (Section 3.3.5, Chapter 3). This can be explained by the fact that plants indirectly respond to herbivory by producing extrafloral nectar to promote the effectiveness of the natural enemies of attacking herbivores (Arimura *et al*. 2009). However, I observed varied nectar production and sucrose concentration changes in the two common bean varieties I used in this study depending on the virus pathogen and growth conditions. There is rich literature on the ability of viral proteins to interfere with plant hormonal signalling pathways to their advantage (Section 3.3.5, Chapter 3). It has been shown that jasmonates and isoleucine–jasmonic acid conjugate subsequently control the secretion of extrafloral nectar in lima bean (Radhika *et al*. 2010). It is highly likely that BCMV and CMV proteins indirectly interfere with nectar production and sucrose concentration by interfering with jasmonates and isoleucine–jasmonic acid conjugate signalling pathways. Further studies are required to investigate how nectar production and sucrose concentration changes come about in the presence of viral pathogens.

7.3.1 Application of induced nectar rewards in agriculture

Plant breeders usually focus on breeding crops for drought resistance, harvest index and pest and disease resistance, and overlook breeding for floral traits such as rewards that influence Pollination services (Richards 2000; Kobayash *et al*. 2010; Tester & Langridge 2010). According to Bailes (2018), selective sweeps or genetic drifts might have contributed to the loss of optimal floral trait combinations that attract pollinators and maintain high pollination rates. Hence the suggestions by Bailes *et al*. (2015), Mallinger & Prasifka (2017), and Bailes *et al*. (2018) that crop breeding through selecting for floral traits could lead to improved food security by attracting greater numbers of pollinators. Bees have a limited diet, and the current declines in populations are partly attributed to the loss of suitable food sources (Goulson *et al*. 2015). Breeding of crop varieties that provide better quality nectar and pollen rewards for bees could improve foraging resources for wild pollinator populations (Palmer *et al*. 2009; Carruthers *et al*. 2017), and in so doing, the amount and variety of food available for humans will be increased. I speculate that by inducing increased nectar production and nectar sucrose concentration in common bean, viruses might be restoring

the original reward status in these crops before they were repeatedly bred. Another possible speculation is that induced improved nectar quantity and quality could be reflective of wild plant behaviour. This could probably because viruses have coevolved with wild plants over long periods and probably viral genes or their products interact with plant genetic pathways to influence nectar production and other rewards.

Future research could focus on investigating how viruses induce changes in nectar rewards in common bean, for example, which specific proteins are involved in promoting nectar production in hosts. A study by Wiesen *et al*. (2016) suggested that in *Arabidopsis thaliana*, GA is involved in the regulation of nectar production. According to Reeves *et al*. (2012), GA induces the expression of jasmonic acid synthesis and response genes in developing stamens. In *Brassica* spp., Radhika *et al*. (2010) showed that floral jasmonic acid levels increase just prior to anthesis and promote nectar secretion. On the other hand, some viruses are known to down-regulate gene expression dependent on jasmonic acid (Lewsey *et al*. 2010; Westwood *et al*. 2014). Thus, viruses interfere with the production or perception of these phytohormones. Hence it would be interesting to investigate the specific pathways that are targeted by viruses, the specific viral proteins involved and how in the bigger picture they influence the production of nectar in hosts.

7.4 Delay in flowering induced by viruses may increase pollination services in hosts

When plants are exposed to stressful conditions, they accelerate their transition to reproduction. Plants growing in drought, heat, low light, low nutrients, overcrowded and shady conditions respond by flowering faster (Casal & Smith 1989; Halliday *et al*. 1994; Martinez-Zapater *et al*. 1994; Levy & Dean 1998; Kazan & Lyons 2016). Given the similarity between responses to abiotic stress and pathogen infection (reviewed in Kazan & Lyons 2016), it is possible that plants also accelerate their reproduction in response to pathogen infection. I speculate that delay in the onset of flowering caused by BCMV and BCMNV (Chapter 3) could be plant response to the presence

of viral pathogens, although whether they do so has not been explored. The rational being susceptible hosts invest more resources towards viral defence by increasing levels of cell death, defence mechanisms characteristic of systemic acquired resistance (Glazebrook 2001), and salicylic acid (O'Donnell *et al*. 2001). Susceptible hosts also exhibit increased levels of auxin, ethylene and JA (Dong 1998; Lund *et al*. 1998; O'Donnell *et al*. 2003). These defence mechanisms are costly to the plant and may impact on onset flowering; hence resource allocation towards flowering may be delayed.

On another note, delay in flowering could be virus-induced, and the ability of viruses to delay flowering abounds in literature (Stein 1962; Shepherd & Purcifull 1971). According to life history evolution models predictions, organisms faced with severe disease should evolve to reproduce more quickly (Minchella 1985; Hochberg 1992; Forbes 1993; Agnew *et al*. 2000). In BCMNV and BCMV-infected plants, the onset of flowering was significantly delayed by about 8-11 days and 2-5 days respectively (Chapter 3). Common bean infected with these two viruses does not behave in the same way as mentioned in the above life history predictions. Hence, I suggest that BCMV and BCMNV induced delay in flowering in common bean, rather than plant response.

Some plants have evolved ways of altering flowering periods to reduce competition for pollinators (Frankie 1975; Anderson & Schelfhout 1980; Berrached *et al*. 2017). It is evident that an advance or delay in flowering may have a pollination advantage over on-time flowering because of increased intensity of pollinator visitation (Eberle *et al*. 2014). This could be true for BCMV that delays flowering by a few days in cv. Wairimu, although flowering will overlap with un-infected plants. Although VOCs from BCMV-infected plants were more attractive to bumblebees, the nectar reward was much greater under poor light conditions and similar to un-infected plants under glasshouse conditions. Thus, delay in flowering might be more beneficial to BCMV-infected plants that offer the same reward as un-infected plants because competition for pollinators may be

avoided for a few days. This will allow the reproduction of BCMV-infected plants on their own for that period where flowering will not be in synchrony with un-infected plants.

On the other hand, BCMNV delayed flowering for a longer period, and there was a shorter period on flowering synchronization with mock-inoculated plants. BCMNV is a virulent pathogen that causes severe. During flowering, BCMNV-infected plants emit VOCs that do not induce innate attraction biases as observed in Chapter 5. In Chapter 6, bees frequently visited more rewarding flowers, a trait that is not induced in BCMNV-infected plants. I speculate that it might benefit BCMNV if it causes changes in flowering time since it may help its susceptible hosts avoid competition for pollination services. Thus, delayed flowering may confer an incidental competitive advantage to susceptible hosts (and an indirect advantage to the virus) (Section 3.3.1, Chapter 3), although I could not test this experimentally.

7.5 Viruses appear to pay back common bean plants by improving their reproductive fitness

Pollination experiments carried out in the glasshouse, and in the University of Cambridge Botanic Garden showed that CMV infection in common bean conferred a reproductive advantage to virus-infected plants because bees visited them more. CMV-infected plants that were not visited by bees yielded significantly lower seed numbers than mock-inoculated plants, but when bees visited their flowers, seed yield was markedly increased to nearly match the seed production in mock-inoculated plants that were not visited by bees. My results provide evidence that in the presence of plant viral pathogens, pollination services may elicit partial yield compensation in common bean. My results also provide evidence that in partially pollinator-dependent and self-compatible crops like beans, bee-pollination services increase seed yield by over 30% (Chapter 6). More than 90% of the leading 109 global crops are pollinated by bees (Greenleaf & Kremen 2006; Klein *et*

al. 2007; Winfree *et al*. 2007). Declining bee populations could threaten global food security as well as the survival of bee-pollinated wild plants (Biesmeijer *et al*. 2006; Potts *et al*. 2010; Abrol 2011; Goulson *et al*. 2015). This information is essential to farmers outside Europe and the USA where crops are pollinated by feral bees and BCMV, BCMNV and CMV are endemic (due to vectors or seed infection) especially in crops thought to self-pollinate. This evidence requires these farmers to rethink on how to use pollinators to improve yields, thus by capitalizing on use of commercial colonies for pollinator services. Thus, even as we seek to secure yields by controlling pests and virus vectors, rescuing yields using pollinators is an additional option.

7.6 Bee pollination of virus-infected plants reduces virus seed transmission rate in common bean.

BCMV-infected plants that were pollinated by common carder bees in the University of Cambridge Botanic Garden produced progeny that had a lower percentage of BCMV seed-transmission when compared with progeny from plants that were produced by BCMNV-parents that self-fertilised. This can be explained by the fact that cross-fertilisation increases heterozygosity in subsequent generations (Ebmeyer 1988; Link *et al*. 1994). Vertical seed-transmission is known to reduce virus accumulation and virulence. Thus plant-virus co-evolution is promoted (Pagán *et al*. 2014). On the other hand, although the rate of vertical seed-transmission is lowered in progeny of BCMV-infected plants, deposition of pollen grains from virus-infected plants onto the stigmas of mock-inoculated plants by bee pollinators increase the chances of cross-pollination. Hypothetically, if this occurs in wild plants, the rate of transfer of alleles conferring virus susceptibility to progeny of recipient plants will be increased. Groen *et al*. (2016) suggested that pollinators can play an important indirect role on the prevalence of CMV and perhaps other viruses in wild plant communities under natural conditions through pollen transfer from virus-infected plants onto healthy.

FAO presented the Quality Declared Seed System guidelines in 1993 and revised them in 2006 (FAO 2006). They aim to provide an alternative for seed quality assurance, which is less demanding than full seed quality control systems (FAO 2006). They were particularly designed for small-scale farmers, agricultural extension services, field agronomists, and specialists in seed production with limited resources, yet guaranteeing satisfactory levels of seed quality (FAO 2006). The reduction of virus seed transmission as observed in bee-pollinated cv. Wairimu and BCMV could be beneficial to seed breeders who are interested in producing virus-free seed. Some farmers in Africa also use saved seed from previous farming season, and this information might be beneficial to them to reduce viral diseases prevalence. Again, capitalizing on use of commercial colonies for pollinator services, together with wild pollinators, could increase yield in beans and reduce the rate of virus seed transmission in saved seed.

7.7 CONCLUSIONS

In conclusion, I have generated evidence that viruses pay back susceptible hosts by attracting pollinators through inducing changes in host emitted VOCs and improving nectar quantity and quality, thereby compensating for yield loss. An improvement in nectar reward for pollinators is a new addition to the payback hypothesis because it extends to benefit the pollinators, thereby promoting cross-pollination in susceptible hosts. I have also provided evidence that viruses improve the reproductive fitness of susceptible hosts in the presence of pollinators. Viruses may be perceived as plant-pollinator mutualism enhancers or mediators because they elicit attraction of pollinators. They can also be thought of as plant mutualists rather than antagonists because they make their hosts more competitive for pollinators. The negatives to this in natural environments are that the delayed flowering caused by BCMNV and to a lesser extent BCMV would lead to accumulation, over time, of virus susceptible plant genotypes. Dishonest signals from virus-infected plants will not attract pollination from foraging bees for long and hence the payback hypothesis will not hold. Also, in the absence of pollinators, this would be severely

deleterious to yields in virus-infected plants. Future work should investigate the mechanisms by which viruses induce changes in VOC emissions and nectar production versus plant response to the presence of viruses. That knowledge and understanding may be useful in agricultural systems.

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APENDICIES

Appendix I: **Primers used in cDNA amplification of BCMV, BCMNV, CMV bean strain in RT-PCR**

Primers used in cDNA amplification of the *Bean common mosaic necrosis virus* PV-0413 isolate

Forward primer	Sequence 5'-3'	Reverse primer	Sequence 5'-3'
CP F	AGGTTAACTCAAAAACCTCA	CP R	AGAGAATATTCATACCCGC

Primers used in cDNA amplification of the *Bean common mosaic virus* PV-0915 isolate

Forward primer	Sequence 5'-3'	Reverse primer	Sequence 5'-3'
CP-END F	TGACAATGGCACTTCACC	CP-END R	AACAAACATTGCCGTAGC

Primers used in cDNA amplification of the *Cucumber mosaic virus* PV-0473 isolate

Forward primer	Sequence 5'-3'	Reverse primer	Sequence 5'-3'
CP F	ACCATCTCCTAGGTTTCTTCGG	CP R	GTCTCCTTTTGGAGGCCC